METHOD OF MODIFYING PLANT PRODUCTIVITY

FIELD OF THE INVENTION

The present invention relates generally to a method of modifying plant productivity comprising expressing in a plant cell, tissue or organ one or more genes capable of facilitating a plant's ability to utilise soil phosphorus. More particularly, the present invention provides a method of increasing plant productivity comprising expressing in the root of a plant an isolated nucleic acid molecule encoding a phytase polypeptide for a time and under conditions sufficient for said phytase to be secreted from the root, and preferably, further comprising modifying the chemistry of the soil around the root using an organic acid. The present invention extends to novel phytase-encoding genes; genetic constructs which are useful for performing the inventive method; and to transgenic plants produced therewith having improved productivity compared to their otherwise isogenic counterparts.

GENERAL

Those skilled in the art will be aware that the invention described herein is subject to variations and modifications other than those specifically described. It is to be understood that the invention described herein includes all such variations and modifications. The invention also includes all such steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

Throughout this specification, unless the context requires otherwise the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps. The present invention is not to be limited in scope by the specific embodiments described herein, which are intended for the purposes of exemplification only. Functionally-equivalent products, compositions and methods are clearly within the scope of the invention, as described herein.

Bibliographic details of the publications referred to by author in this specification are collected at the end of the description.

Reference herein to prior art, including any one or more prior art documents, is not to be taken as an acknowledgment, or suggestion, that said prior art is common general knowledge in Australia or forms a part of the common general knowledge in Australia.

As used herein, the term "derived from" shall be taken to indicate that a particular integer or group of integers has originated from the species specified, but has not necessarily been obtained directly from the specified source.

This specification contains nucleotide and amino acid sequence information prepared using the programme PatentIn Version 2.0, presented herein after the claims. Each nucleotide or amino acid sequence is identified in the sequence listing by the numeric indicator <210> followed by the sequence identifier (e.g. <210>1, <210>2, etc). The length, type of sequence (DNA, protein (PRT), etc) and source organism for each nucleotide or amino acid sequence are indicated by information provided in the numeric indicator fields <211>, <212> and <213>, respectively. Nucleotide and amino acid sequences referred to in the specification are defined by descriptor "SEQ ID NO:" followed by the numeric identifier. For example, SEQ ID NO: 1 refers to the information provided in the numeric indicator field designated <400> 1, etc.

The designation of nucleotide residues referred to herein are those recommended by the IUPAC-IUB Biochemical Nomenclature Commission, wherein A represents Adenine, C represents Cytosine, G represents Guanine, T represents thymine, Y represents a pyrimidine residue, R represents a purine residue, M represents Adenine or Cytosine, K represents Guanine or Thymine, S represents Guanine or Cytosine, W represents Adenine or Thymine, H represents a nucleotide other than Guanine, B represents a nucleotide other than Adenine, V represents a nucleotid

other than Thymine, D represents a nucleotide other than Cytosine and N represents any nucleotide residue.

The designation of amino acid residues referred to herein are also those recommended by the IUPAC-IUB Biochemical Nomenclature Commission, wherein three-letter and one-letter abbreviations for naturally-occurring amino acids are listed in Table 1. In addition to the abbreviations listed in Table 1, the three-letter symbol Asx, or the one-letter symbol B, denotes Asp or Asn; and the three-letter symbol Glx, or the one-letter symbol Z, denotes glutamic acid or glutamine or a substance, such as, for example, 4-carboxyglutamic acid (Gla) or 5-oxoproline (Glp) that yields glutamic acid upon the acid hydrolysis of a peptide.

The designation of plasmid pBS389 herein shall be taken to mean the plasmid depicted in Figure 3, which is also known by those skilled in the art as plasmid pPLEX502, and includes the SCSV promoter and terminator sequences taught in International Patent Application No. PCT/AU95/00552.

Amino acid designations referred to herein are listed in Table 1.

TABLE 1

Amino Acid	Three-letter Abbreviation	One-letter Symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Any amino acid as above	Xaa	X

BACKGROUND TO THE INVENTION

In light of the dwindling supply of land available for agriculture, intensive agriculture production is an imperative for the purposes of feeding the increasing worldwide population. To achieve this end, it is necessary to increase the productivity of agriculture plants. High productivity is of great agricultural and horticultural value, because increased growth reduce times-to-harvest and yield of crop plants. This improvement is of considerable value in the case of both forage and grain crops.

It is well known that phosphorus may boost or even optimise plant productivity. Soil phosphorus may originate from the deposition of organic material in the soil, which form can account for at least 50-85% of total soil phosphorus. However, organic forms of soil phosphorus, such as, for example, inositol phosphorus (soil phytate), may also account for a substantial component of total soil phosphorus. For example, in Australian soils, phytate accounts for up to 38% of total organic phosphorus, and organic phosphorus may account for 50-85% of total soil phosphorus.

Present methods for boosting plant productivity include the application of phosphate-based fertilisers to the soil. High costs of intensive agriculture, particularly in respect of producing agronomically-important crops, are incurred by the requirement to apply phosphate-based fertilisers to the soil. This is especially evident in regions where the soils are deficient in forms of phosphorus that are readily utilisable by plants. Additionally, there is a considerable environmental cost associated with the use of phosphate fertilisers in particular, due to run-off entering the water catchment and resulting in algal blooms under appropriate conditions.

In spite of the benefits to be derived from providing phosphorus to plants in terms of increased productivity, the use of phosphate-based fertilisers has declined recently, in part due to the high economic costs associated therewith and in part due to the high environmental costs. This decline in phosphate-based fertiliser usage has occurred in regions where soils are deficient in forms of phosphate that are

available to plants. This has meant a reduction in plant productivity, particularly in those regions having phosphorus-deficient soils.

Notwithstanding the high proportion of total phosphorus present in the soil in the form of soil phytate, plants almost exclusively derive their phosphorus requirement from soluble phosphate anions, and possess a very limited capacity to directly obtain phosphorus from soil phytate, because phytate is not absorbed by plant roots and further, because phytate is inefficiently hydrolysed to inositol and phosphorus in the soil.

STATEMENT OF THE PRIOR ART

Microorganisms and fungi in the soil are known to contain phytase enzymes that catalyse the conversion of phytate to inositol and inorganic phosphate and phytase-encoding genes of *Aspergillus niger* have been described previously in United States Patent No. 5, 436, 156 issued on 25 July, 1995 (hereinafter "Van Gorcom *et al.*, 1995").

Additionally, Van Ooijen *et al.* (United States Patent No. 5, 593, 963 issued on 14 January, 1997) expressed the *Aspergillus ssp.* phytase gene in the cells of plants, in particular the seeds, with a view to increasing the level of available phosphorus in feedstock containing the transgenic plants. However, the transgenic plants produced by Van Ooijen *et al.* do not possess improved phosphorus nutrition by virtue of an ability to *utilise* soil phytate.

Hayes *et al* (1999) measured the phytase and acid phosphatase activities in root extracts of burr medic, phalaris, white clover, and subterranean clover. These authors concluded that phytase activity was less than 5% of the total acid phosphatase activity in extracts of roots of these plant species. The authors also characterised the biochemical properties of the phytase enzyme in root extracts of a phosphorus-deficient subterranean clover plant , and showed that the enzyme was inhibited by cobalt, zinc, or arsenate, and suggested that cysteine and EDTA may be effective in the chelation of heavy metals that interfere with phytase activity.

Hayes *et al* (1999) concluded that a number of pasture plants have limited ability to use phytate-derived phosphorus as a substrate for growth, consistent with the earlier conclusions of Hübel and Beck (1996) that phytase was unlikely to play a role in the phosphorus nutrition of *Zea mays,* notwithstanding that phytase enzyme and phytate were measurable in the root of that species.

SUMMARY OF THE INVENTION

In work leading up to the present invention, the present inventors sought to improve the phosphorus nutrition and yield of plants without the extensive application of phosphate-based fertilisers, by increasing or improving the ability of a plant to utilise phytate, in particular soil phytate. Surprisingly, the present inventors have found that by ectopically expressing phytase enzyme in the roots, and secreting the phytase into the extracellular environment outside the root, the ability of the plant to utilise phytate as a source of phosphorus is markedly improved. Plants produced according to the inventive method provide considerable benefits to the agriculture sector, in the form of reduced economic and environmental costs, and improved plant productivity relative to their otherwise isogenic counterparts. These benefits are further enhanced if the inventive method is coupled with the step of modifying the chemistry of the soil around the root using an organic acid.

Accordingly, one aspect of the invention provides a method of enhancing the phosphorus nutrition of a plant comprising ectopically expressing in the root of a plant an isolated nucleic acid molecule encoding a phytase polypeptide for a time and under conditions sufficient for said phytase to be secreted from the root.

In a preferred embodiment of the invention, the phytase enzyme is secreted from root cells in the region of the root tip and/or the zone of elongation, that divide more rapidly than those cells that are more distal to the root tip.

Preferably, the secretion of phytase from the root provides a high local concentration of active phytase enzyme in the vicinity surrounding those root cells that are involved in active phosphorus uptake. In work leading up to the present

invention the inventors found that mere cell damage or sloughing that occurs during the movement of the root through the soil fails to provide sufficient phytase activity in the region surrounding those root cells involved in phosphorus uptake, and that an active secretion mechanism is important to achieve the improved phosphorus nutrition of the invention, Accordingly, a preferred embodiment of the present invention provides for the phytase enzyme to be produced as a fusion polypeptide with a secretory signal sequence that is active in plant cells and capable of achieving protein transport not merely outside of a root cell, but outside the root surface. In a particularly preferred embodiment, the phytase enzyme is produced as a fusion polypeptide with the leader sequence of the carrot extensin polypeptide to facilitate extracellular targeting of phytase outside the root surface.

In an alternative embodiment, the present invention provides a method of enhancing the phosphorus nutrition of a plant comprising:

(i) ectopically expressing in the root of a plant an isolated nucleic acid molecule encoding a phytase polypeptide for a time and under conditions sufficient for said phytase to be secreted from the root; and

(ii) modifying the chemistry of the soil around the root or other growth medium around the root using an organic acid, preferably for a time and under conditions sufficient to solubilise phosphorus produced by the action of said phytase enzyme on phytate, or preferably for a time and under conditions sufficient to make the phytate accessible to the phytase enzyme.

The application of the inventive method results in the production of plants having higher biomass production and/or increased phosphorus content compared to otherwise isogenic counterparts, including higher rates of hypocotyl and epicotyl production, leading to a greater accumulation of biomass and larger plants, without the need for extensive application of phosphate-based fertilisers in soils that comprise phytate or to which phytate has accumulated as a result of past agricultural practice. By virtue of its relative insolubility compared to phosphate anions in soil, phytate-based fertilizers may also provide environmental advantages

relative to super-phosphate based fertilizers. It is also known that phytate is abundant in the excreta of animals, particularly monogastric animals, such as, for example, pigs and poultry, wherein animal excreta represent a significant source of phosphorus contamination into the environment. The inventive method described herein provides a significant solution to the problems of using phytate as a fertilizer for plants, from both the perspective of unlocking those phytate reserves in soils and animal excreta, and from the perspective of reducing environmental contamination associated with the reliance upon superphosphates. Accordingly, the present invention clearly provides for the production and use of "phytate-based fertilisers" in conjunction with the inventive method.

Accordingly, a further aspect of the invention contemplates a plant fertiliser comprising phytate and/or a fertiliser composition comprising phytate and a suitable carrier for application to plants and/or the soil.

The present invention further extends to the plants produced by the performance of the inventive method.

A further aspect of the present invention provides an isolated nucleic acid molecule encoding a phytase polypeptide and having more than about 92% nucleotide sequence identity to SEQ ID NO: 1 and/or which is capable of hybridising to SEQ ID NO: 1 or a complementary nucleotide sequence thereto under high stringency hybridisation conditions. Preferably, the isolated nucleic acid molecule of the invention is derived from a microbial source such as, for example, the filamentous fungi *Aspergillus ssp.*

Alternatively, the isolated nucleic acid molecule comprises a nucleotide sequence that encodes an amino acid sequence having more than about 95% identity to the sequence set forth in SEQ ID NO: 2 or an enzymically-active fragment thereof.

This aspect of the invention does not extend to the publicly available *PhyA-1* gen of *Aspergillus niger* (GenBank Accession No. M94550; SEQ ID NO: 3),

notwithstanding that the invention clearly extends to the use of the *PhyA-1* gene or the *PhyA-2* gene in performing the inventive method described herein.

In one embodiment, the isolated nucleic acid molecule encoding phytase is obtainable by the method of:

a) hybridising under at least low stringency conditions plant genomic DNA, RNA or cDNA derived therefrom with one or more nucleic acid probes or primers of at least 10 nucleotides in length for a period of time and under conditions sufficient to form a double-stranded nucleic acid molecule, wherein said probes or primers comprise a nucleotide sequence obtainable from SEQ ID NO: 1 or a nucleotide sequence that is complementary thereto;

b) detecting the hybridised nucleic acid molecule; and

c) isolating said hybridised nucleic acid molecule comprising said genetic sequence.

In a particularly preferred embodiment, the isolated nucleic acid molecule of the invention comprises or consists of the nucleotide sequence set forth in SEQ ID NO: 1 or a fragment thereof encoding an active phytase enzyme. Alternatively, the isolated nucleic acid molecule comprises or consists of a nucleotide sequence that encodes the amino acid sequence set forth in SEQ ID NO: 2 or an enzymically-active fragment thereof.

A further aspect of the present invention extends to gene constructs comprising a phytase-encoding nucleotide sequence connected in-frame to a secretory signal-encoding nucleotide sequence, and placed operably in connection with a promoter sequence that is operable in the root cells of a plant. Preferably, the phytase-encoding nucleotide sequence comprises or consists of the *Aspergillus niger PhyA-2* nucleotide sequence set forth in SEQ ID NO: 1 or a homologue or derivative thereof as described herein.

Preferably, the secretory-signal-encoding nucleotide sequence is the carrot extensin secretory signal or equivalent.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a copy of a representation of a nucleotide sequence alignment between the open reading frames of the chimeric genes produced between the 99 bp nucleotide sequence encoding the carrot extensin leader sequence (bold type) and either the *Aspergillus niger PhyA-1* gene (*PhyA-1.seq;* GenBank Accession No. M94550; SEQ ID NO: 3) or the *A. niger PhyA-2 gene (PhyA-2.seq*; SEQ ID NO: 1) obtained by the present inventors. The alignment was produced using the CLUSTAL W algorithm of Thompson *et al* (1994). Numbering refers to the nucleotide positions from the start of the chimeric *ext::PhyA* genes. Bars between the sequences represent identical nucleotide residues.

Figure 2 is a copy of a representation of an amino acid sequence alignment between two fusion polypeptides comprising the carrot extensin leader sequence (bold type) fused to either the *Aspergillus niger* PhyA-1 polypeptide (*PhyA-1.pro;* GenBank Accession No. M94550; SEQ ID NO: 4) or the *A. niger* PhyA-2 polypeptide (PhyA-2.pro; SEQ ID NO: 2) obtained by the present inventors. The alignment was produced using the CLUSTAL W algorithm of Thompson *et al* (1994). Numbering refers to the amino acid positions from the start of the chimeric polypeptides. Bars between the sequences represent identical amino acid residues.

Figure 3 is a copy of a representation of the pPLEX vector plasmid designated pBS389. This plasmid contains the sub-clover stunt virus (SCSV) region 1 promoter (Sc1 Pr; International Patent Application No. PCT/AU95/00552) and SCSV region 3 terminator (Sc3 3'; International Patent Application No. PCT/AU95/00552) operably connected to a kanamycin-resistance gene (nptII) for expression in plants, flanked by the *Agrobacterium tumefaciens* left-border (LB) and right-border (RB) integration sequences; a bacterial-operable spectinomycin/streptomycin resistance gene (Sp-R/St-R); an *Agrobacterium* origin of replication (oriVRK2) and *E.coli* origin of

replication (oricolE1) and intergenic spacer (IS1/oriT). Positions of restriction sites are indicated.

Figure 4 is a copy of a representation of the plasmid pART 7. Plasmid pART7 is a vector containing bacterial two origins of replication (f1 ori and ori), an ampicillin resistance gene for bacterial selection (Amp^R). Plasmid pART7 also contains two *NotI* restriction sites flanking a CaMV 35S promoter-multiple cloning site(MCS)-NOS 3' cassette, wherein the MCS permits cloning of structural genes in operable connection with said promoter and terminator sequences.

Figure 5 is a copy of a representation of the plasmid pAER02, containing the carrot extensin leader-encoding sequence (ext) in-frame with the *A. niger PhyA-1* gene (PhyA-1; SEQ ID NO: 3) and placed operably in connection with the CaMV 35S promoter sequence and OCS terminator sequence. This vector is based upon plasmid pBS389 (Figure 3).

Figure 6 is a copy of a representation of the plasmid pAER04, containing the carrot extensin leader-encoding sequence (ext) in-frame with the *A. niger PhyA-2* gene (PhyA-2; SEQ ID NO: 1) and placed operably in connection with the CaMV 35S promoter sequence and OCS terminator sequence. This vector is based upon plasmid pBS389 (Figure 3).

Figure 7 is a photographic representation showing the growth of transgenic *Arabidopsis thaliana* (C24 ecotype) plants (T1 generation) containing a binary vector selected from the group consisting of: (i) plasmid pBS389 (row marked "**A**"); (ii) a plasmid containing the *PhyA-2* gene (SEQ ID NO: 1) in plasmid pBS389 (no leader sequence; row marked "**B**"); and (iii) plasmid pAER04 (Figure 6; row marked "**C**"). Transgenic plants were grown for 42 days, at a density of three plants per tube, in sterile agar media supplemented with 0.8 mM Na_2HPO_4 (column marked "Phosphate"), or phytate, equivalent to 0.8 mM phosphate (column marked "Phytate"), or without added phosphorus (column marked "No P"). Duplicate results are indicated for each condition stated herein.

Figure 8 is a photographic representation showing growth on phytate-containing agar media, of transgenic *A. thaliana* (C24 ecotype) plants containing a binary vector selected from the group consisting of: (**A**) plasmid pBS389 ; (**B**) a plasmid containing the *PhyA-1* gene (SEQ ID NO: 3) in plasmid pBS389 (no leader sequence); (**C**) plasmid pAER02 (Figure 5); (**D**) a plasmid containing the *PhyA-2* gene (SEQ ID NO: 1) in plasmid pBS389 (no leader sequence); and (**E**) plasmid pAER04 (Figure 6). Transgenic plants were grown for 42 days, at a density of three plants per tube, in sterile agar media supplemented with phytate, equivalent to 0.8 mM phosphate. Duplicate results are indicated for each condition stated herein.

Figure 9 is a photographic representation showing growth on media lacking added phosphorus (panel **A**), or on phytate-containing agar media having a phytate concentration equivalent to 0.8 mM phosphate (panels **B, C, D**), of transgenic *A. thaliana* (C24 ecotype) plants containing a binary vector selected from the group consisting of: (**A**) plasmid pAER02 (Figure 5); (**B**) a plasmid containing the *PhyA-1* gene (SEQ ID NO: 3) in plasmid pBS389 (no leader sequence); (**C**) plasmid pAER04 (Figure 6); and (**D**) plasmid pAER02 (Figure 5). Plants were grown for 40 days at a density of about thirty plants per agar plate.

Figure 10 is a photographic representation showing growth on sterile agar media plates supplemented with phytate at a concentration equivalent to 0.8 mM phosphate (panels **A, C**), or 0.8 mM Na_2HPO_4 (panel **B**), of transgenic *A. thaliana* (C24 ecotype) plants containing a binary vector selected from the group consisting of: (**A**) a plasmid containing the *PhyA-1* gene (SEQ ID NO: 3) in plasmid pBS389 (no leader sequence); (**B**) plasmid pAER02 (Figure 5); and (**C**) plasmid pAER02 (Figure 5). Plants were grown for 22 days and plates were stained with 0.03% (w/v) $FeCl_3$ to determine regions where phytate was absent from the medium. Regions of darker shading in the plates, in particular the heavier regions along the margins of the roots in Panel **C** show the absence of phytate from the medium, which phytate has been utilised by the plants.

Figure 11 is a photographic representation of a northern blot experiment showing ectopic expression of phytase genes in transgenic *A. thaliana* (C24 ecotype) plants. The upper panel is an ethidium bromide-stained agarose gel containing 10 μg of total RNA isolated from transgenic shoots. The lower panel shows an autoradiograph of the same mRNAs following hybridization under stringent conditions [e.g. at 65°C, in a buffer comprising 0.5xSSC and 0.1% (w/v) SDS] with the *PhyA-1* sequence (SEQ ID NO: 3). The mRNA samples indicated were derived from lines of transformed plants containing a plasmid selected from the group consisting of: (**A**) plasmid pBS389 ; (**B**) a plasmid containing the *PhyA-2* gene (SEQ ID NO: 1) in plasmid pBS389 (no leader sequence); (**C**) plasmid pAER04 (Figure 6); (**D**) a plasmid containing the *PhyA-1* gene (SEQ ID NO: 3) in plasmid pBS389 (no leader sequence); and (**E**) plasmid pAER02 (Figure 5). Duplicate lines are indicated for each plasmid. For RNA isolation, the plants were grown for 36 days on sterile agar supplied with 0.8 mM phosphorus.

Figure 12 is a photographic representation of a Southern blot of total DNA from transgenic *A. thaliana* (C24 ecotype) plants produced using a plasmid selected from the group consisting of: (**A**) plasmid pBS389 ; (**B**) a plasmid containing the *PhyA-2* gene (SEQ ID NO: 1) in plasmid pBS389 (no leader sequence); (**C**) plasmid pAER04 (Figure 6); (**D**) a plasmid containing the *PhyA-1* gene (SEQ ID NO: 3) in plasmid pBS389 (no leader sequence); and (**E**) plasmid pAER02 (Figure 5). DNA samples (~6 μg digested with *Eco*RI) were hybridized under stringent conditions [e.g. at 65°C, in a buffer comprising 0.5xSSC and 0.5% (w/v) SDS] with the *PhyA-1* sequence (SEQ ID NO: 3). Multiple lines are indicated for **C, D,** and **E**. For DNA isolation, the plants were grown for 36 days on sterile agar supplied with 0.8 mM phosphorus. Size markers (kb) are indicated at the left of the Figure. The lane marked # is a control lane containing DNA from non-transgenic *A. thaliana*.

Figure 13 is a photographic representation of a northern blot experiment showing ectopic expression of phytase genes in transgenic *Trifolium subterraneum* (subterranean clover, cultivar Dalkeith) plants (T_0 generation). The upper panel is an ethidium bromide-stained agarose gel containing 10 μg of total RNA isolated

from transgenic shoots. The centre panel shows an autoradiograph of the same mRNAs following hybridization under stringent conditions [e.g. at 65°C, in a buffer comprising 0.5xSSC and 0.1% (w/v) SDS] with the *PhyA-1* sequence (SEQ ID NO: 3). The lower panel indicates the number of transgene inserts as determined by Southern blot analysis (not shown). The mRNA samples indicated were derived from 10 lines of transformed plants, of which five were independent lines (lines **i, ii, iii, iv** and **v**), produced by transformation of explants with the plasmid pAER02 (Figure 5). The lane marked # is a positive control containing RNA from transgenic *A. thaliana* plants produced using the vector pAER02 (*see* Figure 12E). For RNA isolation, tissue culture-derived explant material was grown to maturity under glasshouse conditions.

Figure 14 is a graphical representation showing the release of phosphorus from soil by extraction of air-dried soil in 50 mM citric acid. **Panel (a):**The hydrolysis of organic phosphorus in soil extracts was measured for up to 8 hr in the presence of either no added phytase enzyme (♦), or alternatively, in the presence of different concentrations of commercially-available phytase enzyme, as follows: (a) 0.005 nkat phytase g^{-1} soil (Δ); (b) 0.05 nkat phytase g^{-1} soil (○); or (c) 0.50 nkat phytase g^{-1} soil (■). **Panel (b):**The hydrolysis of organic phosphorus in soil extracts was measured for up to 8 hr in the presence of either no added phytase enzyme (♦), or alternatively, in the presence of different concentrations of purified phytase enzyme, as follows: (a) 0.23 nkat phytase g^{-1} soil (□); or (b) 2.28 nkat phytase g^{-1} soil (■). Soil was from Rutherglen Research Institute, Victoria, Australia, and had been fertilised with superphosphate (9% phosphorus) at a rate of 125 kg ha^{-1} each alternate year since 1914. Time (hr) is indicated on the abscissa, and phosphorus (µg phosphorus (P) g^{-1} soil) is indicated on the ordinate. Each point represents the mean of three observations. Bars indicate standard error and, where not shown, were smaller than the symbol.

Figure 15 is a graphical representation showing the effect of citric acid on the hydrolysis of phytate in air-dried soil, as measured by the releas of phytase-labile organic phosphorus. The hydrolysis of organic phosphorus in soil extracts was

measured for 6 hr in the presence of either 0.50 nkat commercial phytase g^{-1} soil (■), or 2.28 nkat purified phytase g^{-1} soil (□), and various concentrations of citric acid indicated on the abscissa. Phytase-labile organic phosphorus (µg phosphorus (P) g^{-1} soil) is indicated on the ordinate. Total extractable organic phosphorus (µg phosphorus g^{-1} soil) is also shown in the figure (○). Soil was from Rutherglen Research Institute, Victoria, Australia, and had been fertilised with superphosphate (9% phosphorus) at a rate of 125 kg ha^{-1} each alternate year since 1914. Each point represents the mean of three observations. Bars indicate standard error and, where not shown, were smaller than the symbol. Data show a significant positive correlation between the hydrolysis of phytate and the concentration of citric acid in the soil extract.

Figure 16 is a graphical representation showing the effect of pH on the hydrolysis of phytate in air-dried soil, as measured by the release of phytase-labile organic phosphorus. The hydrolysis of organic phosphorus in soil extracts was measured for 6 hr in the presence of either 0.50 nkat commercial phytase g^{-1} soil (■), or 2.28 nkat purified phytase g^{-1} soil (□), in the presence of 50 mM citric acid at the pH values indicated on the abscissa. Phytase-labile organic phosphorus (µg phosphorus (P) g^{-1} soil) is indicated on the ordinate. Total extractable organic phosphorus (µg phosphorus g^{-1} soil) is also shown in the figure (○). Soil was from Rutherglen Research Institute, Victoria, Australia, and had been fertilised with superphosphate (9% phosphorus) at a rate of 125 kg ha^{-1} each alternate year since 1914. Each point represents the mean of three observations. Bars indicate standard error and, where not shown, were smaller than the symbol. Data show that, whilst extractable phosphorus increased with pH, the hydrolysis of phytate by phytase was similar across the range of pH values tested.

Figure 17 is a graphical representation showing the organic phosphorus (filled bars) or inorganic phosphorus (open bars) contents of soils from two sites in Australia that are extractable by water (panels a, b), 50 mM citric acid (panels c, d), or 500 mM sodium bicarbonate (pH 8.5; panels e, f); and the organic phosphorus that was hydrolysed by phytase in soil extracts for 6 hr in the presence of either 0.50 nkat

commercial phytase g^{-1} soil (open-hatched bars), or 2.28 nkat purified phytase g^{-1} soil (densely-hatched bars), in each of the above conditions. One soil sample was from Rutherglen Research Institute, Victoria, Australia, that had been either unfertilised (U_R), or fertilised with superphosphate (9% phosphorus) at a rate of 125 kg ha^{-1} each alternate year since 1914 (F_R), or fertilised as for F_R soils and also top-dressed with nine applications of lime at the rate of 1.25 tonnes ha^{-1} in the period 1914-1948 ($F+L_R$) . The other soil sample was from Ginninderra Experiment Station (Wallaroo 3 paddock) that had been either unfertilised (U_G), or received three autumn applications of triple superphosphate (20.7% phosphorus) at a rate of either 416 kg ha^{-1} ($F1_G$) or 675 kg ha^{-1} ($F2_G$). Extractable phosphorus (µg phosphorus g^{-1} soil) is indicated on the ordinate. Each bar represents the mean of three observations.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

One aspect of the invention provides a method of improving the phosphorus nutrition of a plant comprising ectopically expressing in the root of a plant an isolated nucleic acid molecule encoding a phytase polypeptide for a time and under conditions sufficient for said phytase to be secreted from the root.

As used herein, the term "phosphorus nutrition" shall be taken to refer to the utilisation by a plant of an external source of phosphorus in any form, including organic phosphorus and/or phosphate anion and/or phytate and/or phosphate and/or orthophosphate and/or pyrophosphate, amongst others. By "external source of phosphorus" is meant phosphorus that is taken up by the plant from the external environment.

An "improved phosphorus nutrition" refers to a greater ability of the plant to utilise an existing phosphorus source in the soil or growth medium in which said plant grows.

Accordingly, the present invention is directed to a method of improving the ability of a plant to utilise an external source of phosphorus. In a particularly preferred embodiment, the external source of phosphorus is phytate.

The term "phytate" shall be taken to refer to any storage phosphorus source comprising inositol phosphate, including aggregates and polymers thereof, and phytin, a generic term applied to complex salts of phytic acid (for a review *see* Graf, 1986).

The term "phytase polypeptide" refers to any amino acid sequence, peptide, oligopeptide, polypeptide, or protein molecule, with or without additional non-amino acid substituents or non-naturally-occurring amino acid substituents, that is capable of catalysing the removing a phosphate-containing moiety from phytate as hereinbefore defined. Preferably, a phytase polypeptide will further be capable of catalysing the conversion of phytate to inositol and phosphate, which may be in any form, such as, for example, an anion, or metal complex, a transition metal complex, or a weak acid, amongst others. Those skilled in the art will be aware of those forms of soil phosphate that are readily utilisable by plants, and the present invention clearly extends to phytase enzymes capable of converting phytate to any such form of soil phosphate.

Whilst the present invention is not limited by the source of phytase, the phytase polypeptide of the invention is preferably derived from a plant, microorganism, or animal cell. Those skilled in the art will be aware that phytases are widely-occurring enzymes in nature, derivable from bacteria, such as, for example, *Bacillus subtilis* (Paver and Jagannathan, 1982), *Pseudomonas* (Cosgrove, 1970); yeasts, such as, for example, *Saccharomyces cerevisiae* (Nayini and Markakis, 1984); fungi, such, for example, *Aspergillus fumigatus* (Wyss *et al.*, 1999), Aspergillus *terreus* (Yamada *et al.*, 1986), *A. niger* (Mullaney *et al.*, 1991; van Hartingsveldt *et al.*, 1993); plants (Loewus, 1990) such as, for example, maize (Laboure *et al.*, 1993; Hubel and Beck, 1996; Maugenest *et al.*, 1997), potato (Gellady and Lefebvre, 1990), and soybean (Gibson *et al.*, 1988), amongst others. Conveniently, the phytase enzyme

employed in the performance of the present invention possesses high specific activity and/or high Vmax in a soil environment and/or low Km for phytate in a soil environment.

In a particularly preferred embodiment, the phytase enzyme employed in the performance of the present invention is derived from a fungus, more preferably *Aspergillus spp.*, and even more preferably from *A. niger*. As exemplified herein, the *A. niger* phytase enzymes comprising the amino acid sequences set forth in SEQ ID NO: 2 or SEQ ID NO: 4 provides high biomass production and/or increased phosphorus content when expressed in the roots of transgenic plants and secreted therefrom into the surrounding growth medium.

For the purposes of nomenclature, the amino acid sequence set forth in SEQ ID NO: 2 relates to the *A. niger* PhyA-2 phytase polypeptide, which has been produced by expression of the *A. niger PhyA-2* gene (SEQ ID NO: 1). The present inventors have modified the naturally-occurring gene to be more suitable for expression in plants.

The amino acid sequence set forth in SEQ ID NO: 4 relates to a variant of the *A. niger* PhyA-1 polypeptide (Mullaney *et al.*, 1991; Van Hartingsveldt *et al.*, 1993; GenBank Accession No. M94550), having the leader sequence removed and a different translation start site inserted relative to the naturally-occurring PhyA-1 polypeptide. To express the PhyA-1 polypeptide in plants, the present inventors modified the corresponding *PhyA-1* gene sequence to remove the endogenous *A. niger* leader sequence-encoding nucleotide sequence and intron sequence, and introduced a new translation start site immediately prior to and in-frame with, the nucleotide sequence encoding the mature PhyA-1 polypeptide.

As used herein, the terms "in-frame" and "in the same reading frame" refer to one or more codons of a nucleotide sequence being in the same open reading frame as one or more other codons of said nucleotide sequence. Similarly, the term "in-frame fusion" refers to the linkage between two or more heterologous nucleotide

sequences such that the amino acid sequences encoded thereby are expressed in the same reading frame and, as a consequence, as a single polypeptide molecule.

The phytase polypeptide may be expressed throughout the length of the root, or alternatively, in a localised region of the root, preferably in the zone of elongation and/or the root tip. Conveniently, expression occurs in the epidermal cells and/or the cortex, to facilitate the secretion of the phytase to the root surface, either by reducing the number of cell layers through which the phytase must be transported or alternatively, by facilitating transport of the phytase to the epidermal cells.

Preferably, secretion of the phytase polypeptide from the root cells in which it is expressed is achieved by expressing the phytase as an in-frame fusion polypeptide with a secretory signal sequence capable of directing transport of the phytase to the root surface. According to this embodiment, the secretory signal sequence may be placed at the N-terminal and/or C-terminal end of the phytase polypeptide. Those skilled in the art will be aware that such signal sequences have been demonstrated to be operable in either configuration. Alternatively, or in addition, a secretory signal sequence may be embedded in the phytase polypeptide, the only requirement being that the embedding of the secretory signal sequence in the phytase does not inactivate the phytase enzymic activity in the soil or growth medium. The present invention further encompasses the use of a cryptic secretory signal sequence, either as an in-frame fusion with phytase or alternatively, by mutation of a region of the phytase polypeptide to produce an amino acid variant phytase polypeptide, such as a substitutional variant or insertional variant or deletional variant, that comprises a cryptic secretory signal sequence therein.

Substitutional variants are those in which at least one residue in the phytase amino acid sequence has been removed and a different residue inserted in its place. Amino acid substitutions are typically of single residues, but may be clustered depending upon functional constraints placed upon the polypeptide; insertions will usually be of the order of about 1-10 amino acid residues. and deletions will range

from about 1-20 residues. Preferably, amino acid substitutions will comprise conservative amino acid substitutions, such as those described *supra*.

Insertional amino acid sequence variants are those in which one or more amino acid residues are introduced into a predetermined site in the phytase protein. Insertions can comprise amino– terminal and/or carboxyl terminal fusions as well as intra-sequence insertions of single or multiple amino acids. Generally, insertions within the amino acid sequence will be smaller than amino or carboxyl terminal fusions, of the order of about 1 to 4 residues.

Deletional variants are characterised by the removal of one or more amino acids from the phytase sequence.

Phytase amino acid variants may readily be made using peptide synthetic techniques well known in the art, such as solid phase peptide synthesis and the like, or by recombinant DNA manipulations. The manipulation of DNA sequences to produce variant proteins which manifest as substitutional, insertional or deletional variants are well known in the art. For example, techniques for making substitution mutations at predetermined sites in DNA having known sequence are well known to those skilled in the art, such as by M13 mutagenesis or other site-directed mutagenesis protocol.

Preferred secretory signal sequences according to this embodiment of the invention are derived from plants, fungi, yeasts, bacteria or animal cells, the only requirement being that they function in the root of a plant. Such function can be readily determined without undue experimentation, by determining the level of phytase transported to the cell surface or root surface following its ectopic expression in the root as an in-frame fusion with the secretory signal sequence. Alternatively, or in addition, the efficacy of the signal sequence can be tested by determining the ability of a plant ectopically expressing the phytase as an in-frame fusion with the signal sequence to grow on phytate as a source of phosphorus. As exemplified h r in, and as shown in Figures 7, 8, and 9, plants that ectopically express phytase as an

in-frame fusion with the secretory signal sequence derived from the carrot extensin protein, exhibit improved growth on phytate relative to otherwise isogenic non-transformed plants. Using such tests, those skilled in the art can readily determine the optimum secretory signal sequence for performing the present invention, and the optimum placement of said secretory signal sequence relative to the phytase polypeptide.

The secretory signal sequence is conveniently derived from the full-length potato patatin polypeptide (Iturriaga *et al*,1989; Li *et al.*, 1997), the tobacco PR-S polypeptide (Comelissen *et al.* 1986; Pen *et al.*, 1993), the lupin acid phosphatase (LASAP 1; Wasaki *et al.*, 1999) polypeptide or the carrot extensin polypeptide (Chen and Varner, 1985).

In a particularly preferred embodiment, the secretory signal sequence is derived from the carrot extensin polypeptide and placed at the N-terminus of the phytase polypeptide.

Nucleotide sequences of the secretory signal-encoding nucleotide sequences of the carrot extensin and lupin acid phosphatase genes are set forth herein, as SEQ ID NOs: 5 and 7, respectively. The amino acid sequences of the carrot extensin and lupin acid phosphatase secretory signal peptides are also set forth herein, as SEQ ID NOs: 6 and 8, respectively.

Additionally, the nucleotide sequences of chimeric genes encoding the carrot extensin leader sequence fused to the *PhyA-2* or *PhyA-1* gene, are shown in Figure 1 and SEQ ID Nos: 9 and 11, respectively. The amino acid sequences of fusion polypeptides comprising the carrot extensin leader sequence fused to the modified PhyA-2 or PhyA-1 polypeptides, are shown in Figure 2 and SEQ ID Nos: 10 and 12, respectively. These chimeric genes have been expressd by the inventors in several plant species by the methods described herein to enhance the phosphorus nutrition of those plants.

The word "express" or variations such as "expressing" and "expression" as used herein shall be taken in their broadest context to refer to the transcription of a particular genetic sequence to produce sense or antisense mRNA or the translation of a sense mRNA molecule to produce a peptide, polypeptide, oligopeptide, protein or enzyme molecule. In the case of expression comprising the production of a sense mRNA transcript, the word "express" or variations such as "expressing" and "expression" may also be construed to indicate the combination of transcription and translation processes, with or without subsequent post-translational events which modify the biological activity, cellular or sub-cellular localisation, turnover or steady-state level of the peptide, polypeptide, oligopeptide, protein or enzyme molecule.

Without being bound by any theory or mode of action, the expression of phytase in the root cell and its subsequent secretion to the root surface provides a localised high concentration of active phytase enzyme the is capable of diffusing into the soil to catalyse the conversion of phytate into inositol and phosphate, such that the phosphate is then able to be absorbed or actively taken up by the root.

The term "ectopic expression" refers to the *de novo* and/or increased expression of a peptide, oligopeptide, polypeptide or protein from an introduced nucleic acid molecule, such as, for example, by means of transfection or transformation of a cell, tissue or organ with nucleic acid encoding the peptide, oligopeptide, polypeptide or protein. Ectopic expression can also be achieved by the infection or transformation of a cell, tissue or organ with a foreign organism containing nucleic acid encoding the peptide, oligopeptide, polypeptide or protein.

Accordingly, to ectopically-express a phytase polypeptide comprising an in-frame fusion with a secretory signal sequence, it is necessary to produce a corresponding nucleic acid molecule encoding both the secretory signal sequence and the phytase polypeptide in the same reading frame. This may be achieved by those skilled in the art without undue experimentation.

For the ectopic expression of a peptide, oligopeptide, polypeptide or protein in a plant cell, tissue or organ, the nucleic acid molecule encoding said peptide, oligopeptide, polypeptide or protein in a plant-expressible format, such as, for example, in an appropriate gene construct comprising a promoter sequence operably connected to said nucleic acid molecule, and optionally a transcription termination sequence comprising a polyadenylation signal, amongst others.

By "expressible format" is meant that the isolated nucleic acid molecule is in a form suitable for being transcribed into mRNA and/or translated to produce a protein, either constitutively or following induction by an intracellular or extracellular signal, such as an environmental stimulus or stress (anoxia, hypoxia, temperature, salt, light, dehydration, low phosphate, low P, etc) or a chemical compound such as an antibiotic (tetracycline, ampicillin, rifampicin, kanamycin) hormone (eg. gibberellin, auxin, cytokinin, glucocorticoid, etc), hormone analogue (iodoacetic acid (IAA), 2,4-D, etc) , metal (zinc, copper, iron, etc), or dexamethasone, amongst others. As will be known to those skilled in the art, expression of a functional protein may also require one or more post-translational modifications, such as glycosylation, phosphorylation, dephosphorylation, or one or more protein-protein interactions, amongst others. All such processes are included within the scope of the term "expressible format".

The term "plant-expressible format" refers to an expressible format that pertains to expression of proteins in plant cells, tissues or organs.

Preferably, the ectopic expression of phytase is effected by introducing an isolated nucleic acid molecule encoding phytase, such as a cDNA molecule, genomic gene, synthetic oligonucleotide molecule, mRNA molecule or open reading frame, to a plant cell, tissue or organ, operably in connection with a promoter sequence that is capable of conferring expression in a plant root cell, albeit not necessarily exclusively in the root cell.

Reference herein to a "promoter" is to be taken in its broadest context and includes the transcriptional regulatory sequences derived from a classical eukaryotic genomic gene, including the TATA box which is required for accurate transcription initiation, with or without a CCAAT box sequence and additional regulatory elements (i.e. upstream activating sequences, enhancers and silencers) which alter gene expression in response to developmental and/or external stimuli, or are capable of conferring expression on a structural gene sequence (i.e. the protein-coding region of a gene) in a tissue-specific manner, conveniently in the roots.

In the present context, the term "promoter" is also used to describe a synthetic or fusion molecule, or derivative which confers, activates or enhances expression of a nucleic acid molecule in a plant cell, tissue or organ.

Preferred promoters may contain additional copies of one or more specific regulatory elements, to further enhance expression and/or to alter the spatial expression and/or temporal expression of a nucleic acid molecule to which it is operably connected. For example, copper-responsive, glucocorticoid-responsive or dexamethasone-responsive regulatory elements may be placed adjacent to a heterologous promoter sequence driving expression of a nucleic acid molecule to confer copper inducible, glucocorticoid-inducible, or dexamethasone-inducible expression respectively, on said nucleic acid molecule.

Included within the scope of the present invention is the use of strong constitutive promoter sequences, cell-specific promoter sequences, inducible promoter sequence, tissue-specific promoter sequences, organ-specific promoter sequences, and constitutive promoter sequences that have been modified to confer expression in a particular part of the plant at any one time, such as by integration of said constitutive promoter within a transposable genetic element (*Ac, Ds, Spm, En*, or other transposon).

The term "constitutive" will be known by those skilled in the art to indicate that expression is observed predominantly throughout the plant, albeit not necessarily in

every cell, tissue or organ under all conditions. In the present context, a preferred strong constitutive promoter is one which confers a high level of ectopic expression on a phytase structural gene to which it is operably connected, predominantly throughout the plant and at least in the root, albeit not necessarily in every cell, tissue or organ under all conditions.

The term "cell-specific" shall be taken to indicate that expression is predominantly in a particular plant cell or plant cell-type, albeit not necessarily exclusively in that plant cell or plant cell-type. In the present context, a preferred cell-specific promoter will confer expression on a phytase gene in at least one cell type of the root, preferably, a root epidermal cell or root cortical cell.

Similarly, the term "tissue-specific" shall be taken to indicate that expression is predominantly in a particular plant tissue or plant tissue-type, albeit not necessarily exclusively in that plant tissue or plant tissue-type. In the present context, a preferred tissue-specific promoter will confer expression on one or more tissues of the root, such as, for example, in the zone of elongation, the root vasculature, or the root tip.

Similarly, the term "organ-specific" shall be taken to indicate that expression is predominantly in a particular plant organ albeit not necessarily exclusively in that plant organ. In the present context, a preferred organ-specific promoter will confer expression on a phytase gene throughout the root.

Those skilled in the art will be aware that an "inducible promoter" is a promoter the transcriptional activity of which is increased or induced in response to a developmental, chemical or physical stimulus.

Preferably, the promoter is a root-specific, phosphate-regulated promoter derived from a phosphate transport gene, such as, for example, the phosphate transport genes of *A. thaliana* or barley plants, which are induced under conditions of

phosphate deficiency in the plant. These promoters are obtainable from CSIRO Tropical Agriculture, Queensland, Australia.

The present invention is not to be limited by the choice of promoter sequence and those skilled in the art will readily be capable of selecting appropriate promoter sequences for use in regulating appropriate expression of phytase or modified phytase from publicly-available or readily-available sources, without undue experimentation.

Placing a phytase-encoding nucleic acid molecule under the regulatory control of a promoter sequence, or in operable connection with a promoter sequence, means positioning said nucleic acid molecule such that expression is controlled by the promoter sequence.

A promoter is usually, but not necessarily, positioned upstream, or at the 5'-end, and within 2 kb of the start site of transcription, of the nucleic acid molecule which it regulates.

In the construction of heterologous promoter/structural gene combinations it is generally preferred to position the promoter at a distance from the gene transcription start site that is approximately the same as the distance between that promoter and the gene it controls in its natural setting (i.e., the gene from which the promoter is derived). As is known in the art, some variation in this distance can be accommodated without loss of promoter function. Similarly, the preferred positioning of a regulatory sequence element with respect to a heterologous gene to be placed under its control is defined by the positioning of the element in its natural setting (i.e., the gene from which it is derived). Again, as is known in the art, some variation in this distance can also occur.

Examples of strong constitutive promoters and root-specific promoters that are suitable for use in expressing phytase in the roots of plants include those listed in Table 2, amongst others. The promoters listed in Table 2 are provided for the

purposes of exemplification only and the present invention is not to be limited by the list provided therein. Those skilled in the art will readily be in a position to provide additional promoters that are useful in performing the present invention.

Preferred tissue-specific inducible promoter sequences include the anoxia-inducible and hypoxia-inducible maize *Adh1* gene promoter (Howard *et al.*, 1987; Walker *et al.*, 1987). Such environmentally-inducible promoters are reviewed in detail by Kuhlemeier *et al.* (1987).

Preferred chemically-inducible promoters include the 3-β- indoylacrylic acid-inducible *Tip* promoter; IPTG-inducible *lac* promoter; phosphate-inducible promoter; L-arabinose-inducible *araB* promoter; heavy metal-inducible metallothionine gene promoter; dexamethasone-inducible promoter; glucocorticoid-inducible promoter; ethanol-inducible promoter (Zeneca); the N,N-diallyl-2,2-dichloroacetamide-inducible glutathione-S-transferase gene promoter (Wiegand *et al.*, 1986); or any one or more of the chemically-inducible promoters described by Gatz *et al.* (1996;1998), amongst others.

Preferred wound-inducible or pathogen-inducible promoters include the phenylalanine ammonia lyase (PAL) gene promoter (Ebel *et al.*, 1984), chalcone synthase gene promoter (Ebel *et al.*, 1984) or the potato wound-inducible promoter (Cleveland *et al.*, 1987), amongst others.

In the case of constitutive promoters or promoters that induce expression throughout the entire plant, such sequences may be modified by the addition of nucleotide sequences derived from one or more of the root-specific promoters listed in Table 2, and optionally, additional nucleotide sequences derived from one or more inducible promoters, to confer inducible tissue-specificity thereon. For example, the CaMV 35S promoter may be modified by the addition of maize *Adh1* promoter sequence, to confer anaerobically-regulated root-specific expression thereon, as described previously (Ellis *et al.*, 1987). Such modifications can be achieved by routine experimentation by those skilled in the art.

In a particularly preferred embodiment of the present invention, the phytase is ectopically expressed under control of the CaMV 35S promoter sequence.

In each of the preceding embodiments of the present invention, the phytase protein or a homologue, analogue, or derivative thereof, in particular the *A. niger* phytase protein PhyA-1 or PhyA-2, is expressed under the operable control of a promoter sequence operable in the root. As will be known those skilled in the art, this is generally achieved by introducing a gene construct or vector into plant cells by transformation or transfection means. The nucleic acid molecule or a gene construct comprising same may be introduced into a cell using any known method for the transfection or transformation of said cell. Wherein a cell is transformed by the gene construct of the invention, a whole organism may be regenerated from a single transformed cell, using methods known to those skilled in the art.

By "transfect" is meant that the gene construct or vector or an active fragment thereof comprising the *PhyA-1* or *PhyA-2* gene or a homologue, analogue or derivative thereof, operably under the control of the promoter sequence is introduced into said cell without integration into the cell's genome.

By "transform" is meant that the gene construct or vector or an active fragment thereof comprising the *PhyA-1* or *PhyA-2* gene or a homologue, analogue or derivative thereof, operably under the control of the plant-expressible promoter sequence is stably integrated into the genome of the cell.

In an alternative embodiment, the present invention provides a method of improving the phosphorus nutrition of a plant comprising:

(i) ectopically expressing in the root of a plant an isolated nucleic acid molecule encoding a phytase polypeptide for a time and under conditions sufficient for said phytase to be secreted from the root; and

(ii) modifying the chemistry of the soil around the root or other growth medium around the root.

As used herein, the term "modifying the chemistry of the soil around the root or other growth medium around the root using an organic acid" or similar shall be taken to include any effect of an organic acid on increasing the ability of a plant to utilise phytase-labile phosphorus and/or total organic phosphorus, such as, for example, acidification, and/or a chelating effect that facilitates the solubilisation of phosphorus and/or phosphorus uptake, or to make the phytate accessible to the phytase enzyme, amongst others. The present invention is not to be limited by the mode of action of the organic acid in improving phosphorus nutrition, the only requirement being that the amount of organic acid in the vicinity of the root is increased, such as by direct application, extracellular secretion or active transport, amongst others.

The present invention particularly extends to the use of any agent known to those skilled in the art to modify the chemistry of the soil by chelation, preferably by chelation of a metal, such as, for example a transition metal, to facilitate phosphate uptake by a plant. In this regard, phosphate in the soil which is released by the breakdown of phytate may form complexes with various metals in the soil, such as, for example, aluminium. Without being bound by any theory or mode of action, the presence of an organic acid in the vicinity of the root where phytase acts in accordance with the inventive method may increase access of phytase enzyme to the phytrate substrate by chelation of metal ions, such as, for example, aluminium, iron or calcium, that are known to associate with phytate in the soil. This hypothesis is consistent with data provided as Example 3 herein which show that organic acids increase the availability of organic P substrates and makes them more amendable to dephosphorylation by phytase.

According to this embodiment, the chemistry of the soil around the root may be altered in accordance with the present invention by any means known to those skilled in the art, including the application of organic acids to the soil or growth medium, or the addition of agents known to those skilled in the art that chelate metals but not phosphorus.

However, a particularly preferred means comprises expressing an organic acid biosynthetic enzyme in the roots of the plant so as to increase the intracellular level of organic acids, and the subsequent efflux of organic acids from the root. Preferably, the expression of the organic acid biosynthetic enzyme is targeted to the same cells in which the phytase gene is expressed, to optimise the local extracellular concentrations of available phosphorus in that region of the root which is involved in phosphate uptake.

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referably, the organic acid biosynthesis enzyme is citrate synthase. Expression of citrate synthase may be increased in the region around the root by ectopically-expressing a citrate synthase-encoding nucleic acid molecule in the root under the control of a root-specific promoter sequence or constitutive promoter sequence as described herein, and preferably, targeting the citrate synthase polypeptide product of such expression to the root surface.

As will be apparent to those skilled in the art, the ectopic expression of citrate synthase and targeting of the citrate synthase polypeptide to the root surface may be performed in a similar manner to the expression and secretion of the phytase polypeptide, in accordance with the description provided herein. Preferably, in the performance of this embodiment of the invention, the nucleic acid molecules encoding phytase and the organic acid biosynthesis enzyme are placed operably in connection with different promoter sequences, to minimise competition therebetween for nuclear transcription factors, which competition may reduce expression of one or other structural gene.

Alternatively or in addition, the chemistry of the soil or other growth medium is increased by expressing an organic acid transporter polypeptide in the roots of the plant for a time and under conditions sufficient for the rate or amount of organic acid outside the root to increase.

A further aspect of the present invention clearly provides a gene construct or vector to facilitate the ectopic expression and/or maintenance of the phytase protein-encoding sequence and promoter in a plant cell, tissue or organ.

It will be apparent from the preceding statements that the gene construct of the invention will at least comprise a phytase protein-encoding sequence, optionally further comprising nucleotide sequences encoding a secretory signal sequence operable in plant cells, and a promoter sequence operable in the root cells of a plant operably connected thereto.

The present invention clearly encompasses genetic constructs that further comprise a nucleotide sequence that encodes an organic acid biosynthesis enzyme, in particular citrate synthase, placed operably under the control of a further root-operable or constitutive promoter sequence.

Additionally, the gene construct of the present invention may further comprise one or more terminator sequences.

The term "terminator" refers to a DNA sequence at the end of a transcriptional unit which signals termination of transcription. Terminators are 3'-non-translated DNA sequences containing a polyadenylation signal, which facilitates the addition of polyadenylate sequences to the 3'-end of a primary transcript. Terminators active in cells derived from viruses, yeasts, moulds, bacteria, insects, birds, mammals and plants are known and described in the literature. They may be isolated from bacteria, fungi, viruses, animals and/or plants.

TABLE 2

EXEMPLARY PROMOTERS FOR USE IN THE PERFORMANCE OF THE PRESENT INVENTION

GENE SOURCE	EXPRESSION PATTERN	REFERENCE
root-expressible genes	roots	Tingey *et al.* (1987); An *et al.* (1988);
tobacco auxin-inducible gene	root tip	Van der Zaal *et al.* (1991)
β-tubulin	root	Oppenheimer *et al.* (1988)
tobacco root-specific genes	root	Conkling *et al.* (1990)
B. napus G1-3b gene	root	United States Patent No. 5, 401, 836
SbPRP1	roots	Suzuki *et al.* (1993)
AtPRP1; AtPRP3	roots; root hairs	http://salus.medium.edu/mmg/tierney/html
RD2 gene	root cortex	http://www2.cnsu.edu/ncsu/research
TobRB7 gene	root vasculature	http://www2.cnsu.edu/ncsu/research
Actin	constitutive	
CaMV 35S	constitutive	Odell *et al* (1985)
CaMV 19S	constitutive	
Octopine synthase (OCS)	constitutive	Koncz *et al.*, (1984)
Nopaline Synthase (NOS)	constitutive	Depicker *et al.*, (1982)
gos2	constitutive	de Pater *et al.* (1992)
UBQ1	constitutive	Callis *et al.*, (1990)

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INDUCIBLE PROMOTERS	INDUCTANT	REFERENCE
P5CS (delta(1)-pyrroline-5-carboxylate syntase)	salt, water	Zhang *et al* (1997)
cor15a	cold	Hajela *et al* (1990)
cor15b	cold	Wilhelm *et al* (1993)
cor15a (-305 to +78 nt)	cold, drought	Baker *et al* (1994)
rd29	salt, drought, cold	Kasuga *et al* (1999)
heat shock proteins, including artificial promoters containing the heat shock element (HSE)	heat	Barros *et al* (1992); Marrs *et al* (1993); Schoffl *et al* (1989)
smHSP (small heat shock proteins)	heat	Waters *et al.* (1989)
wcs120	cold	Oullet *et al* (1998)
ci7	cold	Kirch *et al* (1997)
Adh	cold, drought, hypoxia	Dolferus *et al*(1994)
pwsi18	water: salt and drought	Joshee *et al* (1998)
ci21A	cold	Schneider *et al* (1997)
Trg-31	drought	Chaudhury *et al* (1996)
osmotin	osmotic stress	Raghothama *et al* (1993)

\Examples of terminators particularly suitable for use in the gene constructs of the present invention include the *Agrobacterium tumefaciens* nopaline synthase (NOS) gene terminator, the *Agrobacterium tumefaciens* octopine synthase (OCS) gene terminator sequence, the Cauliflower mosaic virus (CaMV) 35S gene terminator sequence, the *Oryza sativa* ADP-glucose pyrophosphorylase terminator sequence (t3'Bt2), the *Zea mays zein* gene terminator sequence, the *rbcs-1A* gene terminator, and the *rbcs-3A* gene terminator sequences, amongst others.

Those skilled in the art will be aware of additional promoter sequences and terminator sequences which may be suitable for use in performing the invention. Such sequences may readily be used without any undue experimentation.

The gene constructs of the invention may further include an origin of replication sequence which is required for maintenance and/or replication in a specific cell type, for example a bacterial cell, when said gene construct is required to be maintained as an episomal genetic element (eg. plasmid or cosmid molecule) in said cell. Preferred origins of replication include, but are not limited to, the *f1*-ori and *col*E1 origins of replication.

The gene construct may further comprise a selectable marker gene or genes that are functional in a cell into which said gene construct is introduced.

As used herein, the term "selectable marker gene" includes any gene which confers a phenotype on a cell in which it is expressed to facilitate the identification and/or selection of cells which are transfected or transformed with a gene construct of the invention or a derivative thereof.

Suitable selectable marker genes contemplated herein include the ampicillin resistance (Amp^r), tetracycline resistance gene (Tc^r), bacterial kanamycin resistance gene (Kan^r), phosphinothricin resistance gene, neomycin phosphotransferase gene (*npt*II), hygromycin resistance gene, β-glucuronidase

(GUS) gene, chloramphenicol acetyltransferase (CAT) gene, green fluorescent protein (*gfp*) gene (Haseloff *et al*, 1997), and luciferase gene, amongst others.

In fact, the phytase protein-encoding sequence may also be used as a selectable marker gene as defined herein, by virtue of the improved phosphorus nutrition of plants secreting phytase from their roots in accordance with the inventive method, including their ability to grow on phytate-containing media. Accordingly, the present invention clearly encompasses the selection of transformed plants by their ability to regenerate on media having phytate as the source of phosphorus.

In a further preferred embodiment, the present invention provides a method of modifying the phosphorus nutrition of a plant comprising:

(i) introducing to a plant cell, tissue or organ a gene construct or vector comprising a nucleotide sequence that encodes phytase in a secretable form, operably in connection with a promoter sequence capable of conferring expression in the roots of a plant;

(ii) regenerating a whole plant therefrom; and

(ii) expressing said phytase in the root or one or more of said cells or tissues thereof such that it is secretable to the surface of the root.

A further aspect of the invention provides a transformed plant ectopically-expressing phytase in secretable form, preferably as an in-frame fusion with a secretable signal sequence.

Means for introducing recombinant DNA into bacterial cells, yeast cells, or plant, insect, fungal (including mould), avian or mammalian tissue or cells include, but are not limited to, transformation using $CaCl_2$ and variations thereof, in particular the method described by Hanahan (1983), direct DNA uptake into protoplasts (Krens *et al*, 1982; Paszkowski *et al*, 1984), PEG-mediated uptake to protoplasts (Armstrong *et al*, 1990), electroporation (Fromm *et al.*, 1985), microinjection of DNA (Crossway *et al.*, 1986), microparticle bombardment of tissue explants or cells (Christou *et al*,

1988; Sanford *et al.*, 1987; Finer and McMullen, 1990; Finer *et al.*, 1992; Sanford *et al.*, 1993; Karunaratne *et al.*, 1996; and Abedinia *et al.*, 1997), vacuum-infiltration of tissue with nucleic acid, or T-DNA-mediated transfer from *Agrobacterium* to the plant tissue (An *et al.*1985; Herrera-Estrella *et al.*, 1983a; 1983b; 1985).

For example, the transformed plants can be produced by the method of *in planta* transformation method using *Agrobacterium tumefaciens* (Bechtold *et al.*,1993; Clough *et al.*,1998), wherein *A. tumefaciens* is applied to the outside of the developing flower bud and the binary vector DNA is then introduced to the developing microspore and/or macrospore and/or the developing seed, so as to produce a transformed seed. Those skilled in the art will be aware that the selection of tissue for use in such a procedure may vary, however it is preferable generally to use plant material at the zygote formation stage for *in planta* transformation procedures.

Alternatively, microparticle bombardment of cells or tissues may be used, particularly in cases where plant cells are not amenable to transformation mediated by *A. tumefaciens*. In such procedures, microparticle is propelled into a cell to produce a transformed cell. Any suitable ballistic cell transformation methodology and apparatus can be used in performing the present invention. Exemplary apparatus and procedures are disclosed by Stomp *et al.* (U.S. Patent No. 5,122,466) and Sanford and Wolf (U.S. Patent No. 4,945,050). When using ballistic transformation procedures, the genetic construct may incorporate a plasmid capable of replicating in the cell to be transformed.

Examples of microparticles suitable for use in such systems include 1 to 5 μm gold spheres. The DNA construct may be deposited on the microparticle by any suitable technique, such as by precipitation.

A whole plant may be regenerated from the transformed or transfected cell, in accordance with procedures well known in the art. Plant tissue capable of

subsequent clonal propagation, whether by organogenesis or embryogenesis, may be transformed with a gene construct of the present invention and a whole plant regenerated therefrom. The particular tissue chosen will vary depending on the clonal propagation systems available for, and best suited to, the particular species being transformed. Exemplary tissue targets include leaf disks, pollen, embryos, cotyledons, hypocotyls, megagametophytes, callus tissue, existing meristematic tissue (e.g., apical meristem, axillary buds, and root meristems), and induced meristem tissue (e.g., cotyledon meristem and hypocotyl meristem).

The term "organogenesis", as used herein, means a process by which shoots and roots are developed sequentially from meristematic centres.

The term "embryogenesis", as used herein, means a process by which shoots and roots develop together in a concerted fashion (not sequentially), whether from somatic cells or gametes.

The generated transformed plants may be propagated by a variety of means, such as by clonal propagation or classical breeding techniques. For example, a first generation (or T1) transformed plant may be selfed to give homozygous second generation (or T2) transformant, and the T2 plants further propagated through classical breeding techniques.

The generated transformed organisms contemplated herein may take a variety of forms. For example, they may be chimeras of transformed cells and non-transformed cells; clonal transformants (e.g., all cells transformed to contain the expression cassette); grafts of transformed and untransformed tissues (e.g., in plants, a transformed root stock grafted to an untransformed scion).

The present invention is applicable to any plant, in a particular monocotyledonous plants or dicotyledonous plant including fodder or forage legume, companion plant, food crop, tree, shrub, or ornamental selected from the list comprising *Acacia spp.*,

Acer spp., Actinidia spp.,Aesculus spp., Agathis australis, Albizia amara, Alsophila tricolor, Andropogon spp., A. thaliana, Arachis spp, Areca catechu, Astelia fragrans, Astragalus cicer, Baikiaea plurijuga, Betula spp., Brassica spp., Bruguiera gymnorrhiza, Burkea africana, Butea frondosa, Cadaba farinosa, Calliandra spp, Camellia sinensis, Canna indica, Capsicum spp., Cassia spp., Centroema pubescens, Chaenomeles spp.,Cinnamomum cassia, Coffea arabica, Colophospermum mopane, Coronillia varia, Cotoneaster serotina, Crataegus spp., Cucumis spp., Cupressus spp., Cyathea dealbata, Cydonia oblonga, Cryptomeria japonica, Cymbopogon spp., Cynthea dealbata, Cydonia oblonga, Dalbergia monetaria, Davallia divaricata, Desmodium spp., Dicksonia squarosa, Diheteropogon amplectens, Dioclea spp, Dolichos spp., Dorycnium rectum, Echinochloa pyramidalis, Ehrartia spp., Eleusine coracana, Eragrestis spp., Erythrina spp., Eucalyptus spp., Euclea schimperi, Eulalia villosa, Fagopyrum spp., Feijoa sellowiana, Fragaria spp., Flemingia spp, Freycinetia banksii, Geranium thunbergii, Ginkgo biloba, Glycine javanica, Gliricidia spp, Gossypium hirsutum, Grevillea spp., Guibourtia coleosperma, Hedysarum spp., Hemarthia altissima, Heteropogon contortus, Hordeum vulgare, Hyparrhenia rufa, Hypericum erectum, Hyperthelia dissoluta, Indigo incarnata, Iris spp., Leptarrhena pyrolifolia, Lespediza spp., Lettuca spp., Leucaena leucocephala, Loudetia simplex, Lotonus bainesii, Lotus spp., Macrotyloma axillare, Malus spp., Manihot esculenta, Medicago sativa, Metasequoia glyptostroboides, Musa sapientum, Nicotianum spp., Onobrychis spp., Ornithopus spp., Oryza spp., Peltophorum africanum, Pennisetum spp., Persea gratissima, Petunia spp., Phaseolus spp., Phoenix canariensis, Phormium cookianum, Photinia spp., Picea glauca, Pinus spp., Pisum sativum, Podocarpus totara, Pogonarthria fleckii, Pogonarthria squarrosa, Populus spp., Prosopis cineraria, Pseudotsuga menziesii, Pterolobium stellatum, Pyrus communis, Quercus spp., Rhaphiolepsis umbellata, Rhopalostylis sapida, Rhus natalensis, Ribes grossularia, Ribes spp., Robinia pseudoacacia, Rosa spp., Rubus spp., Salix spp., Schyzachyrium sanguineum, Sciadopitys verticillata, Sequoia sempervirens, Sequoiadendron giganteum, Sorghum bicolor, Spinacia spp., Sporobolus fimbriatus, Stiburus alopecuroides, Stylosanthos humilis, Tadehagi spp, Taxodium distichum, Themeda

triandra, Trifolium spp., Triticum spp., Tsuga heterophylla, Vaccinium spp., Vicia spp.Vitis vinifera, Watsonia pyramidata, Zantedeschia aethiopica, Zea mays, rice, straw, amaranth, onion, asparagus, sugar cane, soybean, sugarbeet, sunflower, carrot, celery, cabbage, canola, tomato, potato, lentil, flax, broccoli, oilseed rape, cauliflower, brussel sprout, artichoke, okra, squash, kale, collard greens, and tea, amongst others, or the seeds of any plant specifically named above or a tissue, cell or organ culture of any of the above species.

Preferably, the plant is a plant that is capable of being transfected or transformed with a genetic sequence, or which is amenable to the introduction of a protein by any art-recognised means, such as microprojectile bombardment, microinjection, *Agrobacterium-mediated* transformation, protoplast fusion, protoplast transformation, *in planta* transformation, or electroporation, amongst others.

In a particularly preferred embodiment, the transformed plant is *A. thaliana* or subterranean clover. As will be known to those skilled in the art, the provision of both transformed species is sufficient to enable the enhancement of phosphorus nutrition in any plant species using the inventive method described herein.

This aspect of the invention further extends to plant cells, tissues, organs and plants parts, propagules and progeny plants of the primary transformed or transfected cells, tissues, organs or whole plants that also comprise the introduced isolated nucleic acid molecule or gene construct comprising same, and, as a consequence, exhibit similar phenotypes to the primary transformants/transfectants or at least are useful for the purpose of replicating or reproducing said primary transformants/transfectants.

A further aspect of the invention provides an isolated nucleic acid molecule comprising a nucleotide sequence which encodes or is complementary to a nucleotide sequence which encodes a phytase peptide, oligopeptide, polypeptide, protein or enzyme having at least about 93% nucleotide sequence identity to the

Aspergillus niger PhyA-2 gene sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 9 or a complementary nucleotide sequence thereto.

Preferably, the percentage identity to SEQ ID NO: 1 or SEQ ID NO: 9 is at least about 95%, more preferably at least about 97%, and still more preferably at least about 99%. In a particularly preferred embodiment, the isolated nucleic acid molecule of the invention comprises or contains the nucleotide sequence set forth in SEQ ID NO: 1 or SEQ ID NO: 9 or a fragment thereof that encodes an enzymically-functional phytase peptide, oligopeptide or polypeptide.

In determining whether or not two nucleotide or amino acid sequences fall within defined percentage identity or similarity limits referred to herein, those skilled in the art will be aware that it is necessary to conduct a side-by-side comparison. In such comparisons or alignments, differences will arise in the positioning of non-identical residues depending upon the algorithm used to perform the alignment. In the present context, references to percentage identities between two or more sequences shall be taken to refer to the number of identical and similar residues respectively, between said sequences as determined using any standard algorithm known to those skilled in the art. In particular, nucleotide and/or amino acid identities can be calculated using the GAP programme of the Computer Genetics Group, Inc., University Research Park, Madison, Wisconsin, United States of America (Devereaux *et al*, 1984), which utilizes the algorithm of Needleman and Wunsch (1970) or alternatively, the CLUSTAL W algorithm of Thompson *et al* (1994) for multiple alignments, to maximise the number of identical/similar residues and to minimise the number and/or length of sequence gaps in the alignment.

Alternatively or in addition, the present invention encompasses those phytase-encoding nucleotide sequences that are capable of hybridising under high stringency hybridisation conditions to SEQ ID NO: 1 or SEQ ID NO: 9 or a complementary nucleotide sequence thereto, but not including the *PhyA-1* gene sequence.

For the purposes of defining the level of stringency, those skilled in the art will be aware that several different hybridisation conditions may be employed. As used herein, a high stringency may comprise a standard reaction buffer used in a polymerase chain reaction (PCR) to anneal an oligonucleotide primer to template DNA at temperatures higher than 42°C, or alternatively, a standard DNA/DNA hybridisation and/or wash carried out in 0.1xSSC buffer, 0.1% (w/v) SDS at a temperature of at least 65°C, or equivalent annealing/hybridisation conditions.

As will be known to those skilled in the art, the stringency is increased by reducing the concentration of SSC buffer, and/or increasing the concentration of SDS in a standard hybridisation, and/or increasing the temperature of the annealing/hybridisation of PCR or a standard hybridisation, and/or increasing the temperature of the wash in a standard hybridisation. Conditions for hybridisations and washes are well understood by one normally skilled in the art. For the purposes of clarification (to parameters affecting hybridisation between nucleic acid molecules), reference is found in pages 2.10.8 to 2.10.16. of Ausubel *et al.* (1987), which is herein incorporated by reference.

As will be known to those skilled in the art, the specificity of PCR may also be increased by reducing the number of cycles, or the time per cycle, or by the use of specific PCR formats, such as, for example, a nested PCR, a format that is well-known to those skilled in the art. For the purposes of clarification of the parameters affecting the specificity of PCR, reference is made herein to McPherson *et al.* (1991) which is incorporated by way of reference.

Particularly preferred variants of the *A. niger PhyA-2* gene exemplified herein comprise degenerate nucleotide sequences (i.e. homologues) that encode the amino acid sequence set forth in SEQ ID NO: 2.

Preferably, the isolated nucleic acid molecule encodes a phytase polypeptide, protein or enzyme as an in-frame fusion with a secretory signal peptide, in particular the carrot extensin signal peptide.

Homologues, analogues and derivatives of the phytase-encoding nucleotide sequence of the present invention may be obtained by any standard procedure known to those skilled in the art, such as by nucleic acid hybridization (Ausubel *et al*, 1987), polymerase chain reaction (McPherson *et al*, 1991) screening of expression libraries using antibody probes (Huynh *et al*, 1985), and the invention encompasses all such homologues, analogues and derivatives falling within the above-mentioned sequence identity and/or hybridisation limitations.

In nucleic acid hybridizations, genomic DNA, mRNA or cDNA or a part of fragment thereof, in isolated form or contained within a suitable cloning vector such as a plasmid or bacteriophage or cosmid molecule, is contacted with a hybridization-effective amount of a nucleic acid probe derived from SEQ ID NO: 1 for a time and under conditions sufficient for hybridization to occur and the hybridized nucleic acid is then detected using a detecting means.

Detection is performed preferably by labelling the probe with a reporter molecule capable of producing an identifiable signal, prior to hybridization. Preferred reporter molecules include radioactively-labelled nucleotide triphosphates and biotinylated molecules.

Preferably, variants of the *A. niger PhyA-2* gene exemplified herein, including genomic equivalents, are isolated by hybridisation under high stringency conditions, to the probe.

In the polymerase chain reaction (PCR), a nucleic acid primer molecule comprising at least about 14 nucleotides in length derived from the *A. niger PhyA-2* gene is hybridized to a nucleic acid template molecule and specific nucleic acid molecule

copies of the template are amplified enzymatically as described in McPherson *et al*, (1991), which is incorporated herein by reference.

In expression screening of cDNA libraries or genomic libraries, protein- or peptide-encoding regions are placed operably under the control of a suitable promoter sequence in the sense orientation, expressed in a prokaryotic cell or eukaryotic cell in which said promoter is operable to produce a peptide or polypeptide, screened with a monoclonal or polyclonal antibody molecule or a derivative thereof against one or more epitopes of a phytase polypeptide and the bound antibody is then detected using a detecting means, essentially as described by Huynh *et al* (1985) which is incorporated herein by reference. Suitable detecting means according to this embodiment include ^{125}I-labelled antibodies or enzyme-labelled antibodies capable of binding to the first-mentioned antibody, amongst others.

A still further aspect of the present invention provides an isolated or recombinant phytase polypeptide selected from the group consisting of:

(i) a phytase polypeptide having at least 95% amino acid sequence identity to the amino acid sequence set forth in SEQ ID NO: 2;

(ii) a phytase polypeptide encoded by a nucleotide sequence having at least 93% identity to SEQ ID NO: 1;

(iii) fragments of (i) or (ii) that possess phytase enzyme activity;

(iv) in-frame fusion polypeptides comprising (i) and/or (ii) and/or (iii) linked to a secretory signal peptide, in particular the carrot extensin secretory signal peptide.

It will be apparent from the preceding description that a recombinant phytase polypeptide or an in-frame fusion polypeptide comprising same may be produced by standard means by expressing a phytase-encoding nucleotide sequence operably under the control of a suitable promoter sequence in a host cell for a time and under conditions sufficient for translation to occur. Such expression may be carried out in a prokaryotic cell, such as, for example, a bacterial cell. Alternatively, such expression

may be performed in a eukaryotic cell such as an insect cell, mammalian cell, plant cell, fungal cell, or yeast cell, amongst others. In any case, unless the sense molecule is expressed under the control of a strong universal promoter, it is important to select a promoter sequence which is capable of regulating expression in the cell comprising the said nucleic acid molecule in an expressible format. Persons skilled in the art will be in a position to select appropriate promoter sequences for expression of the sense molecule without undue experimentation.

Examples of promoters useful in performing this embodiment include the CaMV 35S promoter, NOS promoter, octopine synthase (OCS) promoter, *A. thaliana SSU* gene promoter, napin seed-specific promoter, P_{32} promoter, BK5-T *imm* promoter, *lac* promoter, *tac* promoter, phage lambda λ_L or λ_R promoters, CMV promoter (U.S. Patent No. 5,168,062), T7 promoter, lacUV5 promoter, SV40 early promoter (U.S. Patent No. 5,118,627), SV40 late promoter (U.S. Patent No. 5,118,627), adenovirus promoter, baculovirus P10 or polyhedrin promoter (U.S. Patent Nos. 5,243,041, 5,242,687, 5,266,317, 4,745,051 and 5,169,784), and the like. In addition to the specific promoters identified herein, cellular promoters for so-called housekeeping genes are useful.

In a preferred embodiment, the recombinant phytase polypeptide is provided in a sequencably-pure format or a pure format substantially free of conspecific proteins.

By "sequencably pure" is meant that the subject polypeptide or a homologue, analogue, derivative or epitope thereof is purified sufficiently to facilitate amino acid sequence determination.

Preferably, said polypeptide or a homologue, analogue, derivative or epitope is at least about 20% pure, more preferably at least about 40% pure, even more preferably at least about 60% pure and even more preferably at least about 80% pure or 95% pure on a weight basis.

For the purposes of describing the present invention in more detail, a plasmid comprising *A. niger PhyA-2* gene as an in-frame fusion with the carrot extensin secretion signal-encoding nucleotide sequence, and operably in connection with the CaMV 35S promoter, was deposited on 23 September, 1999, with the Australian Government Analytical Laboratories (AGAL) at 1, Suakin Street Pymble, New South Wales 2073, Australia, under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure, and accorded AGAL Accession No. NM99/06795. Accordingly, the presently-described invention clearly extends to the use of the deposited plasmid and/or the phytase-encoding portion thereof or variants thereof, with or without the secretory signal-encoding portion of said plasmid, in accordance with the scope of each and every embodiment described herein.

The present invention is further described with reference to the following non-limiting Examples and to the drawings.

EXAMPLE 1

Ectopic expression of phytase under control of the CaMV 35S promoter

I. The *PhyA-2* gene

PCR primers PHYF2 and PHYR3 were used to amplify a derivative of the *PhyA-1* gene from genomic DNA isolated from *Aspergillus niger*, strain ATCC9029. The sequence of the PCR primers (which also contained cloning sites for *Eco*RI and *Cla*I, respectively; underlined lower case) were as follows:

Forward(PHYF2): cgcgaattcATGCTGGCAGTCCCCGCCTCG (SEQ ID NO: 13); and
Reverse(PHYR3): ggcatcgatCTAAGCAAAACACTCCGC (SEQ ID NO: 14)

The amplified *PhyA-2* gene is a modified version of the *PhyA-1* gene that is functional in plants, and contains no leader sequence or first intron; a "new" ATG translation start in the open reading frame, immediately prior to, and in frame with, the nucleotide sequence encoding the mature phytase polypeptide. The sequence

of the reverse primer resulted in the TAG translation stop codon being identical to that in the published *A. niger PhyA-1* gene. The amplified gene has been designated "PhyA-2".

The *PhyA-2* gene has approximately 92% DNA sequence identity to the *PhyA-1* gene. Additionally, there is about 95% amino acid sequence identity between PhyA-1 and PhyA-2 polypeptides (Figures 1 and 2).

The nucleotide sequence of the *PhyA-2* gene is set forth in SEQ ID NO: 1, and the derived amino acid sequence encoded by the *PhyA-2* gene is set forth in SEQ ID NO: 2.

II. The *PhyA-1* gene

The *PhyA-1* gene was originally obtained from Dr Mullaney (United States Department of Agriculture) as a 7.016 kb plasmid (pMD4.21), containing a 2.7 kb *Sph*I clone of the *Aspergillus ficuum* strain NRRL3135 (now termed *A. niger*) *PhyA-1* gene, cloned into the plasmid vector pBR322.

The 2.7 kb insert contained a genomic clone of the *PhyA-1* gene with 5' and 3' flanking sequences, including an *Aspergillus* 5' leader sequence and an intron (102 bp) upstream of the coding region for the mature protein (Genbank Accession No. M94550; Van Hartingsveldt *et al.*,1993).

Modifications were made to the *PhyA-1* gene, to delete the leader sequence and the intron, using PCR. These modifications introduced a "new" ATG translation start in the open reading frame, immediately prior to, and in frame with, the nucleotide sequence for the mature peptide. The sequence of the reverse primer resulted in the TAG translation stop codon being identical to that in the published *A. niger PhyA-1* gene.

The sequence of the PCR primers (which also contained cloning sites for *Eco*RI and *Cla*I, respectively; underlined lower case) to obtain a modified version of the gene that is functional in plants were as follows:

Forward(PHYF2): cgcgaattcATGCTGGCAGTCCCCGCCTCG (SEQ ID NO: 13); and
Reverse(PHYR3): ggcatcgatCTAAGCAAAACACTCCGC (SEQ ID NO: 14)

The nucleotide sequence of the modified *PhyA-1* gene is set forth in SEQ ID NO: 3, and the derived amino acid sequence encoded by this *PhyA-2* gene is set forth in SEQ ID NO: 4.

III. The Carrot extensin secretory signal sequence.

Nucleotide sequences encoding the secretory signal sequence of the carrot extensin gene was amplified using PCR, from plasmid pSEGON, obtained from D. Llewellyn, Commonwealth Scientific and Industrial Research Organisation, Canberra, Australia, which plasmid contains nucleotide sequences encoding the extensin signal peptide upstream of a glucose oxidase-encoding gene.

The PCR primers used to amplify the extensin secretory signal sequence were as follows, wherein cloning sites in the primers are underlined and in lower case:
Forward: gcgtctagagaattcATGGGAAGAATTGCTAG (SEQ ID NO: 15); and
Reverse: cgcggatccgcggccgcAGCTGTGGTTTCGGAAGC (SEQ ID NO: 16).

The amplified product was first subcloned as a *Xba*I/*Bam*HI fragment into plasmid pBSIIKS (Stratagene, Palo Alto, California, USA). The amplified fragment was 99 bp in length and codes for 33 amino acids. The sequence of the leader is set forth in SEQ ID NO: 5.

IV. Ext nsin/Phytase Gen Fusion

In-frame" fusions between the extensin secretory signal-encoding sequence and both the *PhyA-1* and *PhyA-2* genes were generated by ligation between the *Not*I site located at the 3' end of the extension secretory signal-encoding sequence and the *Eco*RI site at the 5' end of the *PhyA-1* and *PhyA-2* genes, respectively. This was achieved by generating blunt (ie., flushed) ends of the restriction enzymes sites prior to ligation.

The junction sequence generated from the extensin/phytase fusion comprised the nucleotide sequence 5'-ACGCTGCCATGCTGGCA-3' (SEQ ID NO: 17), encoding the junction amino acid sequence Thr-Ala-Ala-Met-Leu-Ala (SEQ ID NO: 18), which amino acid sequence comprises two codons derived from extensin, an inserted alanine (encoded by the *Not*I-*Eco*RI fusion), and three codons derived from the phytase genes (*PhyA-1* or *PhyA-2* as appropriate).

Without being bound by any theory or mode of action, the Alanine residue at the extensin::phytase junction provides a suitable site for protease cleavage of the precursor polypeptide to remove the extensin secretory signal sequence.

The extensin/phytase in-frame fusions were generated and verified in plasmid pBSIIKS.

The nucleotide sequence of the *extensin::PhyA-2* chimeric gene is set forth in SEQ ID NO: 9, and the amino acid sequence encoded therefor is set forth in SEQ ID NO: 10. The nucleotide sequence of the *extensin::PhyA-1* chimeric gene is set forth in SEQ ID NO: 11, and the amino acid sequence encoded therefor is set fourth in SEQ ID NO: 12.

V. Construction of vectors for plant transformation

Four gene expression cassettes were produced, comprising the *PhyA-1* gene, chimeric *extensin::PhyA-1* gene, *PhyA-2* gene, and chimeric *extensin::PhyA-2* gene, by subcloning these genes, as *Eco*RI-*Kpn*I fragments, from their plasmid pBSIIKS

vector (see above) into plasmid pART7 (Figure 4), which contains a CaMV 35S promoter and *ocs* terminator.

Accordingly, in the resultant plasmid constructs, the phytase genes were placed in operable connection with the CaMV 35S promoter sequence, to produce the plasmids:

1. *35S+PhyA-1+ocs;*
2. *35S+extensin::PhyA-1+ocs;*
3. *35S+PhyA-2+ocs;* and
4. *35S+extensin::PhyA-2+ocs.*

The four phytase transgenes were then subcloned as *Not*I fragments into the binary vector pBS389 (Figure 3). This vector is suitable for plant transformation and contains the selectable marker *npt*II under control of the SCSV Sc1 promoter and Sc3 terminator sequences. The phytase transgenes were cloned such that their orientation (determining the direction of transcription) was the same as the orientation as the selectable marker.

VI. Plant transformation

The pBS389 vectors containing the various phytase gene constructs were transferred to *Agrobacterium tumefaciens strain* AGL1 using standard, tri-parental mating techniques. These strains were used to transform tobacco, *A. thaliana* and subterranean clover using published protocols. Transformed plants of all three species were generated and have been verified to contain the various phytase transgenes by selection on Kanamycin, PCR and by Southern blot analysis using the *PhyA-1* gene as a hybridisation probe.

Figure 12 shows a representative Southern blot hybridisation to the *PhyA-1* nucleotide sequence under high stringency conditions, of DNAs derived from several lines of *A. thaliana* (C24 ecotype) plants generated using plasmid pBS389, or the *PhyA-2* gene (SEQ ID NO: 1) in plasmid pBS389 (no leader sequence), or plasmid pAER04 (Figure 6), or plasmid containing the *PhyA-1* gene (SEQ ID NO: 3) in plasmid pBS389 (no leader sequence); or plasmid pAER02 (Figure 5). Data indicate the presence of the *PhyA-1*-hybridising signal only in plants produced using the *PhyA-1*-containing or *PhyA-2*-containing gene constructs.

VII. Utilization of organic phosphorus substrates by non-transformed plants

When grown under sterile conditions, wild-type (i.e. non-transformed) *A. thaliana* plants were able to access phosphorus from a range of organic phosphorus substrates, and their growth and phosphorus nutrition were comparable to that for plants supplied with inorganic phosphate. By contrast, wild-type plants were unable to acquire P from phytate. When supplied with phytate, a lower shoot dry weight was observed, and the total phosphorus content of the shoots was only 9.3% of the total phosphorus content of plants supplied with inorganic phosphate as Na_2HPO_4, and not significantly different ($P<0.05$) from plants grown in the absence of external phosphorus.

Similarly, shoot phosphorus concentrations of non-transformed plants supplied with phytate, or alternatively, grown without an external phosphorus source were

significantly lower and these plants exhibited markedly lower shoot to root ratios than plants grown on inorganic phosphate as Na_2HPO_4.

In general, no major differences in root dry weights were observed across the various phosphorus treatments, although lower root mass was evident for plants grown in the absence of phosphorus.

The observed phenotype of plants grown on phytate are indicative of a phosphorus deficiency.

Total extracellular acid phosphomonoesterase activities of between 13.6 and 25.5 mU mg^{-1} dry weight were measured for roots of *A. thaliana* plants, with no significant differences ($P<0.05$) occurring between phosphorus-deficient plant grown on phytate or in the absence of phosphorus, and phosphorus-sufficient plants grown on inorganic phosphate as Na_2HPO_4. Similarly, no differences were observed in total acid phosphomonoesterase in crude extracts prepared from whole roots. In these extracts, phytase activity constituted less than 1% of the total phosphomonoesterase activity, while in separate experiments we were unable to detect extracellular phytase activity.

VIII. Analysis of transformed plants

A) Production of phytase-encoding mRNA

Northern blot hybridisations have been conducted to confirm ectopic expression of the introduced phytase genes transgenic tobacco, *A. thaliana* and subterranean clover plants.

Figure 11 shows a representative northern blot hybridisation to the *PhyA-1* nucleotide sequence, of several lines of transgenic *A. thaliana* (C24 ecotype) plants carrying the *PhyA-2* gene (SEQ ID NO: 1) in plasmid pBS389 (no leader sequence), or plasmid pAER04 (Figure 6), or plasmid containing the *PhyA-1* gene (SEQ ID NO: 3) in plasmid pBS389 (no leader sequence); or plasmid pAER02 (Figure 5). Data

obtained indicated that, in most cases, highest levels of phytase-encoding mRNA were present in plants that contain the phytase genes (*PhyA-1* or *PhyA-2*) fused to the extensin secretory signal-encoding nucleotide sequence, suggesting that these sequences may also increase mRNA stability.

Figure 13 shows a northern blot experiment showing ectopic expression of phytase genes in five independent lines of transgenic *Trifolium subterraneum* (subterranean clover, cultivar Dalkeith; T_0 generation), containing plasmid pAER02 (Figure 5). Data indicate a positive correlation between copy number of the introduced transgene and the level of expression of the chimeric extensin::*PhyA-2* transgene.

b) Phytase enzyme activity

(i) Transformed tobacco plants

Phytase enzyme assays were performed using leaf material derived from transformed tobacco plants.

The phytase activity in leaf extracts of control plants was 0.24 nkat phytase g^{-1} fresh wt (0,04 nkat phytase mg^{-1} protein), compared to 30.4 nkat g^{-1} fresh wt (6.2 nkat phytase mg^{-1} protein) for transformed plants containing the *extensin::PhyA-1* gene construct, representing a 130-fold increase in enzyme activity. Similar results are obtained for phytase assays of roots.

(ii) Transformed subterranean clover plants

Phytase enzyme activity increased in the leaves and roots of transgenic subterranean clover that contain the introduced phytase gene constructs. In particular, the phytase activity in the shoots of primary transformants of subterranean clover generated from tissue culture explant material transformed with plasmid pAER02 was approximately 24-fold that observed in the shoots of control plants transformed with the vector pBS389 (Table 3). In contrast, the phytase activity of transformed plants carrying the *PhyA-1* gene without the extensin signal peptide,

was not significantly different from that of the control plants transformed with pBS389.

TABLE 3

Phytase activity of transgenic subterranean clover plants: T0 generation

Construct	Number of plants	Phytase activity (mU phytase g^{-1} fresh wt)	
		mean	range
control*	3	100.1	35.5 - 190.5
PhyA-1	9	76.8	38.7 - 96.9
ext::PhyA-1	20	2443.5	413.3 - 4346.0

* Control plants were transformed with the binary vector pBS389. The phytase containing lines were transformed with derivatives of pBS389 containing *PhyA-1* either without (*PhyA-1*) or with (*ext::PhyA-1*) the extracellular-targeting sequence from the carrot extensin gene.

The high phytase activity of the T0 plant lines expressing the extensin::PhyA-1 fusion polypeptide was transmissable to the T1 generation, and, for the line designated "iv" there was a 3:1 segregation ratio of high phytase:low phytase, consistent with a single gene insertion in the primary transformant (Table 4). For the line designated "i" there was a 15:1 segregation ratio of high phytase:low phytase, consistent with a double gene insertion in the primary transformant giving rise to that line(Table 4). The number of insertions of the *ex::phyA* gene was confirmed by Southern blot analyses.

TABLE 4

Phytase activity of transgenic subterranean clover plants: T1 population

LINE IV		LINE I	
plant number	phytase activity (mU phytase g^{-1} FW)	plant number	phytase activity (mU phytase g^{-1} FW)
1	34.3	1	1220.9
2	1253.2	2	1840.5
3	110.9	3	1517.6
4	544.9	4	1995.9
5	1327.9	5	1247.2
6	165.5	6	837.5
7	813.3	7	1279.5
8	998.9	8	994.9
9	990.9	9	991.0
10	1545.9	10	577.2
11	238.2	11	861.7
12	94.8	12	1961.6
13	879.9	13	974.7
14	1247.2	14	226.1
15	161.5	15	2036.2
16	1693.2	16	244.2
17	938.4	17	1461.1
18	1495.4	18	750.7
19	871.8	19	379.4
20	1245.2	20	1428.8
control-1[#]	106.9	control-1	197.7
control-2	50.5	control-2	60.5

[#] Control plants were transformed with the binary vector pBS389. The phytase containing lines were transformed with *PhyA-1* containing the extracellular-targeting sequence (*ext::phyA-1*) from the carrot extensin gene.

(iii) Transformed *A. thaliana* plants

We determined the phytase enzyme activity in root extracts for two independently (*a* and *b*) transformed lines of *A. thaliana* (C24 ecotype) for each transgene referred to herein above. The plants were grown for 36 days on sterile agar (~120 plants per replicate sample) containing 0.8 mM inorganic phosphate as . For P determination, the plants were supplied with an equivalent concentration of P as phytate and were grown for 42 days (3 plants tube^{-1}, Figure 3*a-e*).

High levels of phytase activity were observed in soluble root extracts prepared from transgenic lines of *A. thaliana* that ectopically-expressed phytase from either the chimeric *ext::PhyA-1* or *ext::PhyA-2* genes (Table 5). Relative to control plants, the phytase activity of roots from lines generated by transformation with the *ex::PhyA-2* and *ex::PhyA-1* chimeric genes were increased by approximately 20- to 25-fold, and by up to 1500-fold, respectively.

In contrast, no difference in phytase activitity was observed between control plants, and transformed plants produced by transformation with the *PhyA-1* and *PhyA-2* genes (i.e. without the signal transport peptide-encoding gene sequence attached; Table 5). Notwithstanding the absence of a detectable difference in phytase enzyme activity, we could confirm the expression of mRNA encoding phytase in Northern blot experiments (Figure 11).

TABLE 5.

Phytase activity and P nutrition of transgenic *A. thaliana* plants

Transgenic line*		Root phytase (mU mg^{-1} protein)	Total shoot P content† (µg P)	Shoot phosphorus (µg mg^{-1} dry wt)
*Control**	*a*	11.4 [a§]	6.3 [a]	2.6 [a]
	b	9.3 [a]	7.3 [a]	1.9 [a]
PhyA-2	*a*	3.9 [a]	6.7 [a]	2.4 [a]
	b	6.6 [a]	6.9 [a]	2.3 [a]
ext::phyA-2	*a*	224.2 [b]	97.4 [b]	11.2 [b]
	b	432.6 [b]	80.7 [b]	10.2 [b]
PhyA-1	*a*	14.3 [a]	6.4 [a]	2.0 [a]
	b	11.7 [a]	5.9 [a]	3.0 [a]
ext::phyA-1	*a*	992.5 [c]	99.1 [b]	10.4 [b]
	b	14,980.4 [d]	81.9 [b]	11.7 [b]

* Control plants were transformed with pBS389.

†Across all transgenic lines, the mean phosphorus content of shoots from plants that were grown under identical conditions, but were supplied with inorganic phosphate (P_i), or alternatively, that were grown with no added phosphorus, was 79.9 ± 28.7 and 1.1 ± 0.6 µg, respectively.

‡Across all transgenic lines, the mean shoot phosphorus content for control plants supplied with P_i, or alternatively, were grown without added phosphorus (as above), were 10.1± 1.0 and 1.5 ± 0.6 µg mg^{-1} dry weight, respectively.

§ Within each column, values followed by different superscripts are significantly different ($P<0.05$). Phytase activities were log_{10}-transformed prior to analysis.

Seedlings of the selected lines were grown in sterile nutrient solution and phytase activity was determined between days 10 and 12 of growth. Extracellular phytase activity was only detected in transgenic *A. thaliana* that contained gene constructs carrying the leader sequence. Under the assay conditions used, we determined that about 10% of the total soluble root phytase activity in these lines was secreted per day as an extracellular enzyme into the growth medium (Table 6). In contrast,

measurable phytase activity was not secreted from roots of the control plants carrying plasmid pBS389 alone, or from the roots of plants that expressed phytase without the leader sequence attached (Table 6). Accordingly, significant zones of phytate depletion were only observed around the roots of those transgenic lines contianing the chimeric *ext::PhyA-1* or *ext::PhyA-2* genes (Figure 10).

TABLE 6

Extracellular phytase activity of transgenic *A. thaliana* (C24 ecotype) plants.

Transgenic plant line *		Secreted phytase activity (mU)		
		Activity per plant per day	Activity per gram f. wt per day	Activity per ug protein
Control	*a*	< 0.01†	< 1.5	< 0.1
PhyA-2	*a*	< 0.01	< 1.5	< 0.1
Ext::PhyA-2	*a*	0.19 ± 0.04	26.0 ± 4.8	4.5 ± 0.9
PhyA-1	*b*	< 0.01	< 1.5	< 0.1
ext::PhyA-1	*b*	10.07 ± 1.04	1538.0 ± 171.5	79.1 ± 5.1

* The control line was transformed with pBS389.

† Each observation is the mean of 4 replicates and is shown ± 1 standard deviations. Where shown with <, the value was below the indicated limit for detection.

c) ***Plant Growth on phytate-containing media***

The growth responses of transgenic plants in media supplemented with phytate were determined for transformed *A. thaliana* plants.

We compared the growth of transformed *A. thaliana* plants in media containing either no added phosphate, or using 0.8 mM Na_2HPO_4 or phytate (equivalent to 0.8 mM phosphate), as a source of phosphorus. Whilst plants transformed with the pBS389 control plasmid, or expressing the *PhyA-2* gene without the extensin leader sequence, could only grow efficiently in the presence of 0.8 mM Na_2HPO_4 , plants transformed with the gene construct pAER04 (Figure 6) and expressing the *extensin::PhyA-2* fusion protein grew efficiently on phytate as well soluble phosphate as a source of phosphorus. Accordingly, data shown in Figure 7 show that plants ectopically-expressing the extensin-phytase fusion polypeptide grow more rapidly on media containing phytate as a sole source of phosphorus.

We also compared the enhancement of growth, using phytate as a sole source of phosphorus, that was obtainable using the *PhyA-1* or *PhyA-2* genes. As shown in Figure 8, transformed *A. thaliana* plants carrying either the *PhyA-1* or *PhyA-2* genes fused to a nucleotide sequence encoding the carrot extensin leader sequence grew efficiently in media having phytate as the sole source of phosphorus (*see* panels C, E of Figure 8). In contrast, plants expressing the phytase genes without the leader sequence (*see* panels B, D of Figure 8) attached grew poorly on phytate, as did plants not expressing any phytase gene (panel A of Figure 8). Accordingly, both modified phytase genes work equally-well to improve the phosphorus nutrition of plants, however significantly more plant growth was obtained for plants expressing the extensin-phytase fusion polypeptide compared to plants expressing phytase but unable to target the phytase to the extracellular space.

To test the requirement for the extensin leader sequence, we also compared the growth of plants expressing the extensin::PhyA-1 fusion polypeptide on media lacking added phosphorus (Figure 9, panel A), to the growth of plants expressing the

PhyA-1 polypeptide without the extensin leader sequence on phytate (Figure 9, panel B), or plants expressing the PhyA-2 polypeptide on phytate (Figure 9, panel C), or expressing the extensin::PhyA-2 fusion polypeptide on phytate (Figure 9, panel D). After 40 days growth, at a density of about thirty plants per agar plate, significantly enhanced growth was observed on phytate, even for lines wherein the introduced phytase-encoding gene lacked nucleotide sequences encoding the carrot extensin leader sequence, compared to plants grown in the absence of a phosphorus source. However, optimum growth was clearly observed where the extensin leader sequence was present.

To demonstrate that the enhanced growth on phytate is due to enhanced utilisation of phytate, we also stained media plates with 0.03% (w/v) $FeCl_3$ to determine regions where phytate was absent from the medium after 22 days of growth. As shown in Figure 10, staining was observed along the margins of the roots in Panel C, showing the absence of phytate from the medium, which phytate has been utilised by plants expressing the extensin::PhyA-1 fusion protein and grown on phytate, but not soluble phosphorus (0.8 mM Na_2HPO_4). Accordingly, the enhanced nutrition on phytate-containing media is correlated with enhanced phytate utilisation by the transgenic plants.

EXAMPLE 2

Phytase activities of plant roots and localisation of activity

The phytase activities in the roots of a wide range of agriculturally important legume and grass species, including subterranean clover, burr medic, white clover, lucerne, tobacco, *A. thaliana,* wheat, phalaris, ryegrass and danthonia, have been determined.

In summary, phytase activities in extracts prepared from roots of these species ranged between 0.1 and 1.7 nkat g^{-1} root fresh wt (equivalent to 6.0 mU g^{-1} root fresh wt and 102.0 mU g^{-1} root fresh wt , respectively), or alternatively, 0.2 to 1.5 nkat mg^{-1} total protein (equivalent to 12.0 mU mg^{-1} protein and 90.0 mU mg^{-1}

protein, respectively), with levels of activity increased by up to 3.3-fold (9.8-fold on a total root protein basis) when seedlings were grown in conditions of phosphorus deficiency.

In contrast, acid phosphatase activity measured in the same extracts ranged between 20 and 60 nkat g^{-1} root fresh wt (equivalent to 1200 mU g^{-1} root fresh wt and 3600 mU g^{-1} root fresh wt, respectively). Phytase activity was a small component only (less than 5% for the range of species investigated) of the total acid phosphatase activity of plant roots, irrespective of the level of phosphorus nutrition.

The extracellular component of root phytase activity is a minor proportion of the total phytase activity measurable in the roots of naturally-occurring plants. For example, less than 0.042 nkat phytase activity g^{-1} root fresh wt (ie., less than 4.7% of the activity measured in root extracts) could be eluted from roots of subterranean clover. The estimated extracellular root phytase activity of intact roots of subterranean clover seedlings is as low as 0.03 nkat g^{-1} root fresh wt (ie., 3% or less of the activity measured in soluble root extracts).

However, supplementation of subterranean clover roots with phytase, at a rate that is equivalent to 0.13 nkat g^{-1} root fresh wt, resulted in a significant enhancement of the ability of the plants to acquire phosphorus from phytate.

EXAMPLE 3

Effect of organic acids on the phytase-labile component of soil phosphorus

We have also demonstrated that the efficacy of organic acids in improving the extractability of phosphorus from the soil, and to show that a significant component of the organic phosphorus in soil actually extracted by citrate is amenable to dephosphorylation by phytase.

Phytase-labile organic phosphorus was determined in various extracts prepared from two Australian pasture soils with contrasting fertiliser histories.

Materials and Methods

Soils

Soil samples were collected to a depth of 10 cm, under permanent pastures (containing perennial grass and annual legume components) located at two sites: (i) Rutherglen Research Institute, Victoria; and (ii) Ginninderra Experiment Station, Canberra, ACT. Individual cores (2.5 cm diameter) of soil (~30 samples) from each treatment at each site were bulked as a composite sample which was air-dried, passed through a 2 mm sieve to remove large, particulate matter and stored at room temperature.

Samples from the Rutherglen site were collected in 1993 from treatments of a non-replicated fertiliser trial, the details for which are published in Ridley *et al.* (1990). Briefly, the trial was established in 1914 on three 1.5 ha fields. Two fields (F_R and $F+L_R$) received approximately 125 kg ha^{-1} of single superphosphate (9% phosphorus) each alternate year from 1914 to 1986, while the third field (U_R) was unfertilised. One of the fertilised fields ($F+L_R$) was also top-dressed with lime from 1914 to 1948, receiving nine applications of 1.25 t ha^{-1}.

Samples from Ginninderra Experiment Station (Wallaroo 3 Paddock) were collected in October, 1996. Three phosphate fertiliser treatments were imposed across the trial on its establishment in 1994. Plots were either unfertilised (U_G) or received three autumn applications of triple superphosphate (20.7% phosphate), totalling 416 kg ha^{-1} ($F1_G$) or 675 kg ha^{-1} ($F2_G$).

Properties of the two soils are presented in Table 7. Total inorganic phosphorus and organic phosphorus were determined by the ignition-extraction (0.5 *M* H_2SO_4) procedure (Olsen and Sommers 1982) and Colwell phosphorus by a 16 h extraction with 0.5 *M* $NaHCO_3$ (Colwell 1963), followed by determination of inorganic phosphorus using the molybdate-blue colour reaction (Murphy and Riley 1962). Organic carbon was determined by the Modified Mebius procedure (Nelson and Sommers 1982) and soil pH was measured in $CaCl_2$.

Substrate specificities of phytase preparations

The substrate specificities of commercial preparations of wheat germ acid phosphatase (EC 3.1.3.2; Sigma Chemical Co.), *Aspergillus niger* phytase (EC 3.1.3.8; Sigma) and a purified preparation of the *A. niger* NRRL 3135 phytase (kindly provided by Dr Markus Wyss; F. Hoffmann-La Roche, Switzerland) were determined using a range of organic phosphorus compounds. The specific activities of the three enzyme preparations were tested at 27 °C, in 50 m*M* MES buffer (pH 5.5) containing 1 m*M* EDTA, against the following substrates: *myo*-inositol hexaphosphoric acid (dodecasodium salt; IHP), α-D-glucose 1-phosphate (disodium salt; G1P), ribonucleic acid (type VI; RNA), adenosine-5'-triphosphate (ATP), D(-)3-phosphoglyceric acid (trisodium salt; PGA), *p*-nitrophenyl phosphate (disodium salt; *p*NPP), and *bis*(*p*-nitrophenyl) phosphate (sodium salt; *bis*-*p*NPP). With the exception of ATP (Boehringer Mannheim), all substrates were obtained from Sigma Chemical Co. Assays were performed in 1 ml volumes, using 50 µg ml^{-1} acid phosphatase; 1.14 µg ml^{-1} commercial phytase; or 0.45 µg ml^{-1} purified phytase. These amounts of enzyme were chosen on the basis of the reported specific activities of the preparations, against either IHP or *p*NPP. Enzyme assays were conducted over 30 min at a range of substrate concentrations between 2.4 m*M* and 4.8 m*M* phosphorus, with three replicates. Activities were measured against *p*-nitrophenol standards (Bessey et al. 1946) for the *p*NPP and *bis*-*p*NPP samples, and for all other samples by the release of inorganic phosphorus as determined using the malachite-green reaction (Irving and McLaughlin 1990).

Table 7

Properties of two pasture soils (0 to 10 cm depth) from Ginninderra and Rutherglen

Site	Soil Type (Stace et al. 1968)	Fertiliser treatment[a]	phosphorus (mg kg^{-1} soil; ignition/extraction method)			pH ($CaCl_2$)	Organic C (%)	Colwell phosphorus (mg kg^{-1} soil)
			Total phosphorus	Inorganic phosphorus	Organic phosphorus			
Ginninderra Experiment Station	Yellow podzolic	U_G (Unfertilised)	203	53	150	4.67	1.88	11.3
		$F1_G$ (86 kg P ha^{-1})	242	79	163	4.57	1.91	25.0
		$F2_G$ (140 kg P ha^{-1})	254	97	157	4.57	1.95	43.7
Rutherglen Research Institute	Grey-brown to yellow podzolics	U_R (Unfertilised)	154	30	123	4.60	1.99	7.8
		F_R (~450 kg P ha^{-1})	312	113	199	4.21	3.07	38.8
		$F+L_R$ (~450 kg P ha^{-1} and 11.25 tonnes lime ha^{-1})	287	95	192	4.55	2.57	26.4

[a]**Total amount of fertiliser applied since inception of trial**

PCT/AU00/01183

Measures of enzyme-labile soil

Soil extraction and total, organic and inorganic phosphorus determinations

Amounts of air-dried soil of between 2 and 10 g were extracted in 50 ml polypropylene tubes using two volumes of sterile extractant solution, usually deionised water, 50 m*M* (~1.0%) citric acid (pH ~2.3) or 0.5 *M* Na-bicarbonate (pH 8.5). Other extractants included 0.025 and 0.1 *M* HCl, and 0.01 *M* $CaCl_2$ (pH 5.5). The soils were extracted at 22 ± 2 °C for 30 min on a reciprocal shaker (300 rpm), followed by centrifugation (10, 300 *g*) for 15 min. Soil extracts were decanted from the pelleted material and stored at 4 °C prior to enzyme analyses.

The phosphorus contents of soil extracts were determined on 1 ml sub-samples of each solution. Inorganic phosphorus was determined by measuring the inorganic phosphorus content of solutions with malachite-green reagent (Irving and McLaughlin 1990). In order to determine total phosphorus, the samples were autoclaved (120 kPa/ 121 °C; 40 min) in the presence of 0.6 *M* H_2SO_4 and 3.3% ammonium persulphate (Schoenau and Huang 1991), and were similarly analysed for inorganic phosphorus content. Corrections for volume loss during autoclaving were made as necessary, based on gravimetric analyses. Organic phosphorus in the extracts was calculated by deduction of inorganic from total phosphorus.

Incubation of soil extracts with enzyme

Standard assays involved incubation of 1 ml of soil extract in the presence of excess enzyme: either 0.25 nkat (ie., 0.50 nkat g^{-1} soil) of commercial phytase, as determined against IHP substrate for the incubation conditions specified herein, or 1.14 nkat (2.28 nkat g^{-1} soil) of purified phytase. These amounts of enzyme equated to 1.56 (3.12 nkat g^{-1} soil) and 0.03 nkat (0.05 nkat g^{-1} soil) of acid phosphatase activity as determined against *p*NPP, for the commercial and purified preparations, respectively. Samples were adjusted to pH ~5.5 with either diluted HCl or NaOH, 300 µl of 250 m*M* MES buffer (pH 5.5; containing 5 m*M* EDTA) was added, and the solutions were made up to a final volume of 1.5 ml with deionised

water. Immediately on addition of the enzyme to each reaction, an aliquot was removed and mixed with a one-fifth volume of 25% trichloro-acetic acid (TCA). Remaining solutions were incubated at 27 °C for 6 h, after which TCA was added to terminate the reaction. The TCA-treated samples were centrifuged in an Eppendorf microfuge (12,000 rpm, 10 min) prior to analysis for inorganic phosphorus using malachite-green reagent. Control treatments were routinely included, whereby either soil extract or enzyme were omitted.

Statistics

Enzyme assays and other soil measures were replicated three times and the data were analysed to investigate variation associated with soil extraction and incubation procedures, using one- and two-way analyses of variance (ANOVAs). Where F-ratios were significant ($P<0.05$), treatment means were compared by least significant difference (LSD).

Results

Substrate specificities and specific activities of phytase preparations

The specific activities of the three enzymes for IHP ranged from 1.0 nkat mg^{-1} protein (wheat bran acid phosphatase) to 560.9 nkat mg^{-1} (purified *A. niger* phytase; Table 8). The purified *A. niger* phytase preparation had a 12-fold higher specific activity for IHP than the commercial phytase. Moreover, purified phytase showed a narrow substrate specificity, with specific activities for a range of organic phosphorus substrates that were 10% or less than the specific activity for IHP. The commercial *A. niger* phytase preparation was less substrate-specific, with highest activity observed against *p*NPP. The activity profile of the commercial phytase preparation was similar to that of wheat bran acid phosphatase.

It is likely that the commercial *Aspergillus* phytase preparation contained a considerable amount of phosphatase activity that was not specific for phytate. This was removed from the more purified preparation. The wheat bran acid

phosphatase was not used in subsequent experiments because of its similarity to commercial phytase.

TABLE 8

Substrate specificities of various phytase enzyme preparations

Substrate	Specific Activity (nkat mg^{-1} protein)		
	Acid phosphatase	Sigma phytase	Purified phytase
myo-inositol hexakisphosphate	1.0	46.9	560.9
Glucose 1-phosphate	0.4	84.8	0.1
Ribonucleic acid	0.4	38.5	0.3
Adenosine triphosphate	4.2	55.2	59.4
Phosphoglyceric acid	3.6	78.7	54.5
p-nitrophenyl phosphate	7.0	293.0	13.1
bis(*p*-nitrophenyl) phosphate	0.2	11.5	35.5
LSD (*P*=0.05)	0.3	14.5	51.7

Inorganic and organic phosphorus contents of extracts from unfertilised Ginninderra soil

Unfertilised soil from Ginninderra Experiment Station (U_G) was selected initially for phosphorus measurements because of its low phosphorus status (Table 7). There was a requirement for soil extracts to contain sufficient levels of organic phosphorus, so that any change upon incubation with enzyme would be detectable. Various extracts of field-moist U_G soil were prepared, in which recoverable organic phosphorus was determined. Water, 0.01 *M* $CaCl_2$, 0.025 *M* HCl and 0.1 *M* HCl extracted less than 1.0 µg organic phosphorus g^{-1} soil. By contrast, up to 22.5 µg phosphorus g^{-1} soil was extracted with citric acid and 0.5 *M* Na-bicarbonate. Na-

bicarbonate, citric acid and water were thus selected as extractants for further analysis. A solution-to-soil ratio of 2:1 extracted higher concentrations of organic phosphorus (as compared to a 5:1 ratio) and was adopted for the subsequent incubation experiments.

Enzyme-labile phosphorus in extracts from fertilised Rutherglen soil.

Fertilised Rutherglen soil (F_R) was used to determine the appropriate conditions to be employed for enzyme incubation studies with all soil-extractant combinations. The hydrolysis of organic phosphorus from extracts of soil F_R was measured for up to 8 h in the presence of three concentrations of commercial or purified phytase, or in the absence of enzyme.

The release of inorganic phosphorus from incubated citric acid soil extracts is shown in Figure 14. Additions of 0.50 nkat commercial phytase or 2.28 nkat purified phytase g^{-1} soil were required for reactions to approach completion within 8 h. With lower amounts of enzyme, reactions proceeded more slowly and did not reach completion during the incubation period. For all subsequent measures of labile phosphorus, including various soils containing lower organic phosphorus concentrations, soil extracts were incubated for 6 h in the presence of 0.50 or 2.28 nkat phytase g^{-1} soil, using commercial or purified preparations, respectively.

To determine the contribution of soil microbe-derived enzyme activities to the observed rates of hydrolysis in amended soil extracts, duplicate samples were passed through 0.45 µm filters (Millex-HA, Millipore). There were no differences in either the rate or total quantity of inorganic phosphorus liberated from citric acid, water or Na-bicarbonate extracts of soil F_R, between filtered and unfiltered samples (data not shown). Therefore, over the 6 h incubation period, microbial enzyme activity did not contribute to the observed rates of hydrolysis. A low, and insignificant, lev l of hydrolysis was measured when no enzyme was added to the extracts (Figure 14).

Effects of citric acid concentration and pH on phytase-labile phosphorus from fertilised Rutherglen soil

Citric acid concentration

Extractable organic phosphorus from soil F_R increased significantly with increasing concentrations of citric acid to 50 m*M* ($P<0.05$; Figure 15). The amounts of organic phosphorus that were hydrolysed by the commercial and purified phytases, and the proportion of the total organic phosphorus which was enzyme-labile, also increased with citric acid concentration. For the commercial phytase, labile phosphorus represented 13.5% and 82% of the organic phosphorus in water and 50 m*M* citric acid soil extracts, respectively. Purified phytase-labile organic phosphorus was approximately half of that hydrolysed by the commercial enzyme preparation, representing between 1.8% and 44.7% of the total organic phosphorus across the same range of citric acid concentrations.

Effect of pH of citric acid

Soil extractions were prepared using 50 m*M* citric acid solutions adjusted to between pH 2.3 and pH 6.0. Extractable organic phosphorus increased with pH, from 7.5 phosphrous g^{-1} soil at pH 2.3 to 25.3 µg phosphorus g^{-1} soil at pH 6.0 (Figure 16). However, enzyme-labile phosphorus was similar across the entire pH range, with ~7.8 µg phosphorus g^{-1} soil and ~4.5 µg phosphorus g^{-1} soil hydrolysed by commercial phytase and purified phytase, respectively (Figure 16).

Comparison between citric and hydrochloric acids as extractants

Total organic phosphorus, and commercial and purified phytase-labile organic phosphorus in water, citric acid and hydrochloric acid extracts from soil F_R are presented in Table 9.

TABLE 9

Extractable organic phosphorus (P_o), and enzyme-labile phosphorus (μg g^{-1} soil) using two sources of phytase from *A. niger*, in water, citric acid and HCl extracts of the fertilised Rutherglen soil (F_R).

n.d. = not detectable.

Extractant	Organic P (μg g^{-1} soil)	Enzyme-labile P_o (μg g^{-1} soil)			
		Commercial phytase (% of P_o)		Purified phytase (% of P_o)	
Water	2.10	0.26	(12.4)	0.07	(3.3)
50 m*M* citric acid (pH 2.3)	7.65	6.15	(80.4)	3.35	(43.8)
50 m*M* HCl (pH 1.45)	1.01	0.09	(8.9)	n.d.	-
5 m*M* HCl, pH 2.3	2.09	0.17	(8.1)	0.07	(3.3)
LSD (*P*=0.05)	0.54	0.30		0.57	

Two HCl extractants were used: one at the same molar concentration as citric acid (50 m*M*) and the other at the same pH (pH 2.3, at ~5 m*M*). When used at an equivalent molar concentration, HCl extracted only 13% of the quantity of organic phosphorus extracted with citric acid, and the enzyme-labile phosphorus component was negligible relative to that of citric acid extracts. When the concentration of HCl was adjusted for a solution of pH 2.3, the amount of extractable organic phosphorus was increased, but was still only 27% of that extracted by citric acid. Likewise, the enzyme-labile organic phosphorus component was less than 3% compared to citric acid. While commercial and purified phytase-labile organic phosphorus in 50 m*M* citric acid extracts represented 80% and 44% of the extractable organic

phosphorus, respectively, less than 13% and 4% of organic phosphorus in the other extracts were hydrolysed by either of the enzyme preparations (Table 9).

Extractable and phytase-labile organic phosphorus from soils with different fertiliser histories

The total and organic phosphorus contents of water, 50 m*M* citric acid (pH ~2.3), and 0.5 *M* Na-bicarbonate (pH 8.5) extracts of soil from phosphorus fertiliser trials at two sites are illustrated in Figure 17, along with the fractions of extractable organic phosphorus that were hydrolysed by commercial and purified phytase during separate incubations.

While Na-bicarbonate extracted more organic phosphorus than the other solutions (between 12 and 30 μg g^{-1} soil; Figure 17, panels *e*, *f*), citric acid extracted the most enzyme-labile organic phosphorus (up to 5.7 μg phosphorus g^{-1} soil; Figure 17*c*, *d*), with between 56% and 79% of the organic phosphorus in citric acid extracts being hydrolysed by the commercial phytase preparation. In contrast, only 7 to 17% and 2 to 9% of the organic phosphorus in water and Na-bicarbonate extracts, respectively, was hydrolysed by the commercial phytase (Figure 17*a*, *b*; Figure 17*e*, *f*). A smaller component of the organic phosphorus was hydrolysed by purified phytase. In citric acid extracts, 28 to 40% of the organic phosphorus was purified phytase-labile (Figure 17*c*, *d*), while only 3 to 8% and 1 to 2% of the organic phosphorus in water and Na-bicarbonate extracts, respectively, was hydrolysed (Figure 17*a*, *b*; Figure 17*e*, *f*).

Organic, inorganic and enzyme-labile phosphorus extracted by citric acid was significantly higher ($P<0.05$) in the fertilised Rutherglen soils (F_R and $F+L_R$) than the unfertilised soil (U_R; Figure 17*c*). In addition, both total extractable organic phosphorus and the component which was phytase-labile, were greater for the fertilised soil (F_R) than soil which had received both fertiliser and lime ($F+L_R$). While organic and inorganic phosphorus extracted by Na-bicarbonate were significantly greater ($P<0.05$) for both fertilised Rutherglen soils, only the treatment which had

received fertiliser alone contained a higher level of extractable, commercial phytase-labile phosphorus than the unfertilised control (Figure 17*e*). No differences in fertiliser treatment were evident for purified phytase-labile phosphorus.

For the soil from Ginninderra, which had received only recent applications of phosphorus fertiliser, there were no differences in either extractable organic phosphorus or enzyme-labile phosphorus across fertiliser treatments (Figure 17). However, the extractable inorganic phosphorus component increased significantly ($P<0.05$) with phosphorus fertility, in both citric acid and Na-bicarbonate extracts (Figure 17*d*, *f*).

Discussion

Characterising phytase-labile organic phosphorus in extracts of soil

Two *A. niger* phytase preparations with markedly different substrate specificities were used to measure the amounts of enzyme-labile organic phosphorus present in extracts of soil. A commercial preparation of *A. niger* phytase (from Sigma Chemical Co.) showed activity against a range of organic phosphorus substrates, with highest activity for *p*NPP. In accordance with previous reports (Wyss et al. 1999), a purified form of the *A. niger* phytase was highly specific for phytate. The contrasting substrate specificities of the two phytase preparations were exploited to characterise the enzyme lability of organic phosphorus extracted from soils. Material hydrolysed by the purified phytase was considered likely to be indicative of the phytate content of the extracts, whereas hydrolysis by the commercial "phytase" preparation reflected a more general acid phosphatase activity. However, it should be noted that the activities of the two preparations were not entirely mutually exclusive, as shown by their activities against a range of organic phosphorus substrates (Table 8).

In water extracts from soils collected from Ginninderra Experiment Station and Rutherglen Research Institute, less than 8% of the organic phosphorus present was hydrolysed by the purified phytase and only 7 to 17% was hydrolysed by the

commercial preparation (Figure 17). Previous measures of enzyme-labile organic phosphorus in water extracts of soils have not been made using phytase with a narrow specificity for IHP.

The present work suggests that low levels of phytate occur in soil solution (less than 0.22 µg phosphorus g^{-1} soil), and also that organic phosphorus esters accessible to a more general phosphatase (ie. commercial phytase) activity are present in only small quantities. In contrast, higher amounts of organic phosphorus in water extracts (between 42 and 70%; Pant et al. 1994) and soil solution (48 to 62%; Shand and Smith 1997) from Scottish soils, were hydrolysed by a phytase (from wheat bran) which was not specific for phytate.

Several extractants were used to assess enzyme-labile organic phosphorus. Sodium-bicarbonate has previously been considered to extract a component of soil organic phosphorus which is readily mineralisable (Bowman and Cole, 1978). On this basis, sequential soil organic phosphorus extraction procedures have been developed to include bicarbonate-extractable organic phosphorus as a labile fraction (Hedley et al. 1982; Sharpley 1985). However, the present experiments do not support the supposition that bicarbonate-extractable organic phosphorus is labile. While Na-bicarbonate extracted the largest quantities of organic phosphorus relative to other soil extractants (up to 30 µg phosphorus g^{-1} soil), only a small proportion (between 1 and 9%) was enzyme-labile, even when using phytase with activity against a wide range of phosphate esters (Figure 17). Otani and Ae (1999) have similarly shown that negligible amounts of organic phosphorus were enzyme-labile in Na-bicarbonate extracts from a range of soils.

In contrast, 50 m*M* citric acid extracted intermediate amounts of organic phosphorus (3.8 to 7.7 µg phosphorus g^{-1} soil), of which up to 79% could be hydrolysed by the commercial phytase preparation and up to 40% was hydrolysed by the purified preparation. Otani and Ae (1999) also used a range of extractants and found that only citrate extracted organic phosphorus that was readily accessible to either acid

phosphatase or broad-specificity phytase. From the present work, it is evident that citric acid and Na-bicarbonate extracts contain different components of the total organic phosphorus pool.

Up to 40% of citrate-extractable soil organic phosphorus was hydrolysed by the purified phytase preparation (Table 9; Figure 17), indicating that a considerable amount may occur as phytate. Citrate is an effective chelator of trivalent metal ions such as Fe^{3+} and Al^{3+} (Jones and Darrah 1994). In soils, citrate can release inorganic phosphorus into solution either by anion exchange with Fe- and Al-associated phosphates on soil adsorption surfaces (Gerke 1992), or by chelation of precipitates to form soluble compounds (eg. Gardner et al. 1983). Phytate undergoes similar adsorption and precipitation reactions in soils to produce inorganic phosphorus (Ognalaga et al. 1994). It is conceivable that soil phytate is released into solution in the presence of citric acid via similar mechanisms.

Citrate also extracted organic phosphorus that was readily accessible to a phytase preparation possessing general acid phosphatase activity (Figure 17). The purified and commercial phytase preparations showed markedly different specific activities against a range of phosphate esters (Table 8). However, it was evident that they also hydrolysed a common component of the citrate-extractable organic phosphorus; when combined, the amounts of extractable organic phosphorus hydrolysed by the two enzymes often exceeded the total quantity of extractable soil organic phosphorus (Figure 17). Enzyme-labile organic phosphorus in citric acid extracts may represent a component of soil phosphorus that can potentially be used by plants. In previous work, we have observed that plants grown in sterile culture were able to use organic phosphorus substrates, such as glucose 1-phosphate and β-glycerophosphate, essentially as equivalent phosphorus sources to inorganic phosphorus for growth, while phytate was a relatively poor source of phosphorus (JE Hayes et al. submitted to *Plant and Soil*, AE Richardson, PA Hadobas and JE Hayes submitted to *Plant, Cell and Environment*).

Higher concentrations of citric acid extracted more soil organic phosphorus and also increased the proportion of organic phosphorus that was enzyme-labile. By contrast, HCl (used at the same molarity as citric acid) extracted minimal phytase-labile organic phosphorus. Consequently, the chelating properties of citric acid, rather than acidification effects, were considered to be largely responsible for the extraction of enzyme-labile organic phosphorus. The amount of extractable organic phosphorus also increased with the pH of the citric acid extractant. It is likely that this was due to the greater chelation ability of the $citrate^{2-}$/$citrate^{3-}$ species, which would predominate over citrate/$citrate^{-}$ species at the higher pH. Gerke and Meyer (1995) found that citrate adsorption to a humic podzol was higher at pH 6.5 compared to pH 5.5. Concurrently, they observed greater mobilisation of phosphorus. In the present work, the amount of enzyme-labile organic phosphorus did not increase with higher citric acid pH.

Effect of fertiliser history on extractable, phytase-labile soil organic phosphorus

Measures of phosphorus in extracts of soils with contrasting fertiliser histories indicated that there were marked differences in the amounts of both total organic phosphorus and enzyme-labile organic phosphorus. Similar amounts of organic and hydrolysed phosphorus were observed in fertilised and unfertilised soils from plots at a recently established field site (Ginninderra). Only the inorganic phosphorus content of the soil at this site had been increased by the relatively recent phosphorus fertiliser applications. By contrast, clear distinctions could be made between fertiliser treatments for soil with a long history of fertiliser application, but which had received no fertiliser for seven seasons prior to sampling (Rutherglen). Differences between phosphorus treatments were especially evident in citric acid extracts, where the extracts from the fertilised soil contained approximately 3-fold greater amounts of enzyme-labile organic phosphorus than extracts from unfertilised soil. Extracts of soil which had received both fertiliser and lime similarly contained higher levels of total organic phosphorus and enzyme-labile organic phosphorus. These results may indicate that, with extended periods of

fertiliser application, a greater proportion of applied phosphorus accumulates in soil as labile organic phosphorus. This component of labile organic phosphorus may make an important contribution to the phosphorus nutrition of pastures. Mineralisable fractions of soil organic phosphorus have been reported to be important for phosphorus cycling within permanent pasture systems (McLaughlin et al. 1990).

Conclusions

We have shown that a only small component of the organic phosphorus in water and Na-bicarbonate extracts of soil can be hydrolysed by phytase and general acid phosphatase activities. This may indicate that only low levels of potentially plant-available organic phosphorus occur in soil solution. By contrast, greater amounts of enzyme-labile organic phosphorus were extracted using citrate. Citrate is exuded into the rhizosphere by roots of a number of plant species and, in many instances, exudation is enhanced under conditions of phosphorus deficiency (eg. Lipton et al. 1987; Hoffland et al. 1989; Grierson 1992; Keerthisinghe et al. 1998). Citrate exudation by plant roots is considered to be an important mechanism for increasing the acquisition of soil inorganic phosphorus. Our results imply that citrate also increases the availability to plants of soil organic phosphorus, by solubilising a fraction that can be hydrolysed by enzymes.

Enzyme-labile soil organic phosphorus was also influenced by soil fertiliser history. While inorganic phosphorus was the dominant labile fraction in soils which had received recent applications of fertiliser, in soils of low fertility (eg. soil U_G) or with a long history of fertiliser application (Rutherglen soil), the enzyme-labile component of citrate-extractable organic phosphorus was equivalent to, or exceeded the quantity of extractable inorganic phosphorus.

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